Population Structure of *Staphylococcus aureus* in China

Xiaomei Yan

ISBN: 978-90-367-8057-5 (printed version)
ISBN: 978-90-367-8056-8 (electronic version)

Printing
Printed by Yangguang printing company

Cover
Cover designed by Ziyu Liu and Xiaomei Yan. The cover is a minimum spanning tree of *S. aureus* isolates from China and Europe based on the relatedness between MLVA types. The right corner is a very famous Chinese traditional painting “the magpie stands in plum” which was painted by Xiaomei’s mother.

university of groningen

Population Structure of *Staphylococcus aureus* in China

PhD thesis

to obtain the degree of PhD at the
University of Groningen
on the authority of the
Rector Magnificus Prof. E. Sterken
and in accordance with
the decision by the College of Deans.

This thesis will be defended in public on

Wednesday 2 September 2015 at 14.30 hours

by

Xiaomei Yan

born on 28 November 1974
in Jilin, China

Supervisors

Prof. H. Grundmann

Prof. J.M. van Dijl

Assessment committee

Prof. R. Köck

Prof. A. Voss

Prof. E. Feil

Paranymphs: Monika Chlebowicz
Eleni Tsompanidou
Corinna Glasner

To my family

献给我的家人

The work described in this thesis was performed in the laboratory of Molecular Bacteriology, Department of Medical Microbiology of the University Medical Center Groningen and University of Groningen, Groningen, the Netherlands and Department of Diagnosis for Communicable Disease, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, China with the support of the National Natural Science Foundation of China (Grant No. 81301463), of the State Key Laboratory for Infectious Disease Prevention and Control Fund of China (2012SKLID202), and of an educational grant from the Graduate School of Medical Sciences of the University of Groningen, the Netherlands.

Printing of this thesis was financially supported by the Graduate School for Drug Exploration (GUIDE) and the University of Groningen, Groningen, the Netherlands.Their support is highly appreciated.

Table of contents

Chapter 1

General Introduction and Scope of this Thesis

Staphylococcus aureus

S. aureus was first described in the 1880s (75). Taxonomically, the genus *Staphylococcus* belongs to the bacterial family *Staphylococcaceae*. Although more than 20 species of *Staphylococcus* are described in Bergey's Manual (7), *S. aureus* is still the most important with regards to its disease-causing potential in both humans and animals. *S. aureus* is a Gram-positive spherical bacterium, 0.4-1.2µm in diameter, which occurs in microscopic clusters resembling grapes (hence the genus name; from Greek *Staphyle* = bunch of grapes) which, in contrast to other *Staphylococci*, form fairly large yellow colonies on rich medium (hence the species name; from Latin *aurum* = gold, Figure 1A, 1B and 1C). By testing the coagulation of rabbit serum, *S. aureus* can be readily distinguished from other *Staphylococcus* species, which are coagulase-negative.

S. aureus is a common commensal of humans and can colonize multiple sites of the human body, but the moist and lower temperature environment of the squamous epithelium of the anterior nares appears to be the main ecological niche. In healthy Caucasians it is estimated that 20% are persistent carriers (104,113). For most healthy individuals, colonization is unproblematic. However, if *S. aureus* contaminates a breach in the skin or mucous membranes, it can go on to infiltrate the underlying tissue causing an infection. There are three lines of evidence that support the view that *S. aureus* nasal colonisation is associated with a higher chance to develop staphylococcal infections. First, the rates of infection are higher in persistent carriers than in other individuals (125). Second, high resolution molecular typing using pulsed-field gel electrophoresis (PFGE) has shown that infecting strains of *S. aureus* were indistinguishable from carriage isolates previously isolated from the external nares of patients who later developed an invasive infection (106,114). Finally, eradication of this microorganism is regarded as an effective means for reducing infections in surgical and dialysis patients (50,125).

S. aureus can cause different diseases. Most frequently, it causes skin and soft tissue infections and infections of the respiratory tract. However, *S. aureus* may also cause a variety of other sometimes very severe and life-threatening conditions, such as infective endocarditis, toxic shock syndrome (TSS), scalded skin syndrome, osteomyelitis, necrotizing pneumonia, disseminating metastatic abscess formation and septic shock. Furthermore, *S. aureus* is a frequent cause of biofilm-associated infections, in particular those developing on indwelling medical devices (76). When people ingest toxinogenic strains of *S. aureus* with contaminated food, food poisoning is common. *S. aureus* is usually transmitted by direct contact with a

colonized or infected individuals, although contact with contaminated objects and surfaces might also play a role in transmission (64).

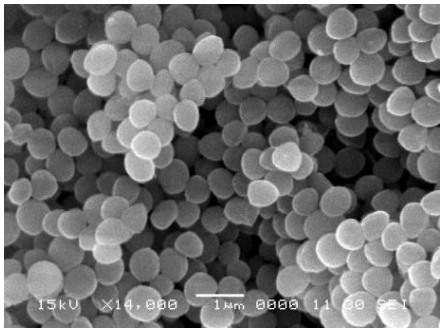

Figure 1A. *Staphylococcus aureus* Scanning Electron Micrograph

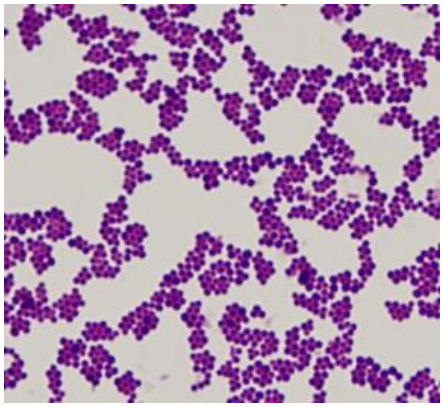

Figure 1B. *Staphylococcus aureus* micrograph by Gram-staining (×1,000)

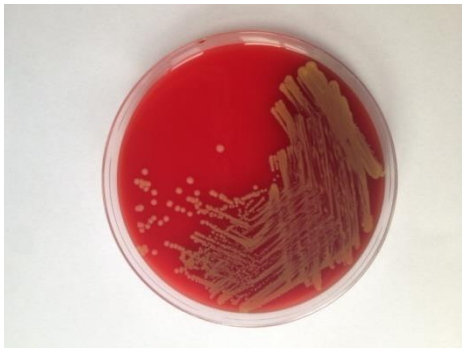

Figure 1C. *Staphylococcus aureus* on blood agar medium.

S. aureus has probably been one of the important pathogens in hospitals especially among surgical patients in human history. Its recent notoriety is to a large extent the result of quickly

accumulating multiple antibiotic resistances. Whenever a new antibiotic was marketed, *S. aureus* always became resistant within contemporaneous time scales (Table 1). Thus, in 1942, 2 years after the introduction of penicillin into clinical practice, the first penicillin-resistant *S. aureus* isolate was observed in a hospital and then spread to the community. In 1961, 2 years after the introduction of methicillin, a penicillinase-resistant penicillin, *S. aureus* developed resistance due to acquisition of the *mecA* gene (5,78), which encodes a modified penicillin-binding protein (PBP2a) with low affinity for all beta-lactam antibiotics. A distinctive feature of methicillin-resistant *S. aureus* (MRSA) strains is their resistance not only to all beta-lactam antibiotics, but also to a wide range of other antibiotics, which makes MRSA infections difficult to treat and control. Since the 1970s, MRSA became firmly associated with hospital-acquired infections worldwide and was hence

designated as hospital-associated MRSA (HA-MRSA). Since the early 1990s, community-associated MRSA (CA-MRSA) lineagues have emerged in on different continents. All were characterized by the presence of the toxin Panton-Valentine leukocidin (PVL) and a non-multi-drug-resistance phenotype (4, 69,83,100). In 2002, the first vancomycin-resistant *S. aureus* (VRSA) was isolated in the United States of America (USA), which is of great concern because of its complete resistance to almost all antibiotics (Centers for Disease Control and Prevention (CDC), 2002).

Apart for human beings, livestock animals including cows, sheep, goats, poultry and rabbits represent another important reservoir. MRSA from animals was first reported in 1972, following detection in milk from cows with mastitis (20), which was considered a major economic threat to the global dairy industry (9). A recently described lineage, clonal complex 398 (CC398), has emerged in the 2000's among livestocks and began colonizing and infecting humans (3,101). With the global spread of this clonal complex, it has become imperative to better understand the evolution, reservoirs and routes of transmission of CC398.

Many factors seem to contribute to the success of *S. aureus* as a pathogen. In addition to its ability to persist as a commensal and acquire resistance to multiple antimicrobial agents, the diverse repertoire of virulence determinants was the third important factor. Superantigen (SAg) toxins currently include enterotoxins, exfoliative toxins A and B and toxic shock syndrome toxin (TSST-1) toxins, which activate T lymphocytes hijacking the natural antigen

Table 1.Milestones in the discovery and treatment of *Staphylococcus aureus*

Year	Event
1880	ander Ogston identifies clustered micrococci in purulent infections
1931	Association between nasal colonisation and furunculosis discovered
1934	Introduction of the coagulase test for the identification of *S. aureus* into clinical
1940	Penicillin introduced
1942	Penicillin-resistant *S. aureus* appears
1944	Introduction of phage typing
1952	Association between nasal colonisation of *S. aureus* and infection with the same
1959	Methicillin introduced
1961	1st clinical cases with MRSA reported
1972	MRSA from animals reported
1991	Pulsed-field gel electrophoresis used for genotyping *S. aureus*
1994	Identification of microbial surface components recognising adhesive matrix
2000	Multilocus sequence typing developed for studying the clonality of *S. aureus*
2001	Whole genome of *S. aureus* sequenced
2001	80% of bacteraemic *Staphylococcus aureus* isolates are endogenous
2001	Increase in community-onset MRSA infections
2002	Vancomycin-resistant *Staphylococcus aureus* reported
2005	ST398 MRSA reported from Dutch farm animals

presentation mechanism (45). *S. aureus* produces a variety of cytolytic toxins as well. These include alpha-toxin (6), the Panton-Valentine leukocidin (PVL, consisting of the LukS and LukF proteins), the leukocidins LukDE and LukAB (LukGH), and gamma-toxin (gamma-hemolysin, HlgA, HlgB, HlgC), which represent cytolytic toxins that can destroy red and/or white blood cells (77). Recently, novel cytolytic peptides, phenol-soluble modulins (PSMs), were suggested to play a multi-functional role in staphylococcal pathogenesis (111).

Global epidemiology of *S. aureus* and MRSA

Hospital or healthcare-associated MRSA (HA-MRSA) are a loosely defined group of clonal lineages that typically cause hospital-acquired infections, whereas community-associated MRSA (CA-MRSA) infections are made up of different lineages and occur in individuals with no previous history of hospitalization. Livestock associated MRSA (LA-MRSA) is a new MRSA variant that has been spreading in animals, especially in pigs and veal calves.

HA-MRSA

The HA types of MRSA are highly prevalent in hospitals worldwide. Data from the 2004 and 2007 LEADER Program recorded a prevalence of 54.2% and 58.1% in the USA, respectively (21,48). Although still a common and severe threat to patients, invasive MRSA infections in healthcare settings appear to be declining in the USA. Between 2005 and 2011, the overall rates of invasive MRSA dropped by 31%; the largest decline (54%) was observed among infections occurring during hospitalization (CDC, Threat Report 2013). MRSA proportions observed in Canada (in 2008) and Australia (in 2011) were 27.0% and 30.3%, respectively (15,126). In Europe, MRSA proportions are generally lower in Northern Europe and higher in the South and South-Eastern countries. The average proportion of MRSA isolated from invasive infection was 17.8% in 2012 (EARSS Annual Report. 2012). Only two countries reported proportions above 50%, which were Portugal (53.8%) and Romania (53.9). The majority of the countries, 19 of 30, reported 10%-50%. Lower proportions were reported in six countries, all less than 3% including Denmark (1.3%), Finland (2.1%), Iceland (1.7%), the Netherlands (1.3%), Norway (1.3%) and Sweden (0.7%).

Currently, more than 50% of *S. aureus* isolates show resistance to methicillin in most countries in Asia. Very high rates of MRSA were reported from East Asian countries, such as Korea (77.6%), Taiwan (65.0%), Hong Kong (56.8%), Thailand (57.0%), Vietnam (74.1%), but also Sri Lanka (86.5%) (94). Iran reported 43.5% *S. aureus* isolates being MRSA (2). In contrast, much lower proportions were reported from India (22.6%) and the Philippines (38.1%).

The prevalence of MRSA was lower than 50% in most of the African countries, although it appears to have risen since 2000 in many African countries, except for South Africa (26). In Tunisia, the prevalence of MRSA increased from 16% to 41% between 2002 and 2007, while in Libya it was 31% in 2007. In Botswana, the prevalence varied from 23–44% between 2000 and 2007. In Algeria and Egypt, the prevalence was 45% and 52% between 2003 and 2005, respectively. In Nigeria, the prevalence was higher in the Northern than the Southern part. In

Ethiopia and the Ivory Coast, the prevalence was 55% and 39%, respectively. In South Africa, the prevalence decreased from 36% in 2006 to 24% during 2007–2011.

MRSA is one of the leading causes of nosocomial infection in the Latin American region. Data revealed an increase in the proportion of MRSA in Latin American medical centers from 33.8% in 1997 to 40.2% in 2006. The following rates of MRSA in 2006 were reported Argentina, 51%; Bolivia, 55%; Brazil, 54%; Chile, 29%; Ecuador, 25%; Mexico, 32%; Panama, 28%; Paraguay, 30%; Uruguay, 24%; and Venezuela, 27% (39).

Statistically significant decreasing trends were observed in some European courtiers, (Belgium, Croatia, France, Germany, Hungary, Ireland, and United Kingdom), the USA, South Africa, and China in recent years. Arguably, this has been caused by improved infection control as well as the restriction of some antibiotic classes in the hospitals globally (99,107).

The majority of MRSA infections are caused by strains belonging to a few clonal complexes (CC) in hospitals between 1961 and 2008. The most prevalent are CC5 (USA100 or NY/Japan clone, USA800 or pediatric clone, UK-EMRSA-3, Italian/Southern German clone), CC8 (Iberian clone or UK-EMRSA-5 or Rome, Brazilian or Hungarian clone, and USA500 or UK-EMRSA-2/6), CC22 (UK-EMRSA-15), CC30 (USA200, UK-EMRSA-16, USA1100, the Southwest Pacific Oceania clone), and CC45 (USA600, Berlin clone) (Figure 2). In summary, CC5 and CC8 are the most abundant clones worldwide. Two pandemic HA-MRSA clones, namely multilocus sequence type (ST) 239 (CC8) and ST5 (CC5), have been spreading in many countries in Asia. CC22 (ST22) was occasionally reported in several Asian countries, such as Malaysia (32,56), Singapore (98) and India (90). CC22 (ST22), CC5 (ST5) and CC8 (ST8) are common in Europe (36). On the other hand CC30 (ST36) used to be common in the UK. The CC5 (ST5), CC8 (ST239) and CC30 MRSA clones predominate in Latin America (86). The five most widely distributed clones in Africa are ST88, CC5 (ST5), CC8 (ST8), ST80 (CC80), and CC8 (ST239/241) (87). CC5 which is named CMRSA2 in Canada (73) and USA100/800 in the US (46,62) is the predominant HA-MRSA clone in North America. Previously the CA-MRSA USA300 has been introduced into hospitals in the USA, where it has become the second most common clone. Around 98.8% of the HA-MRSA isolates were classified as either ST22-IV (EMRSA-15) or ST239-III (Aus-2/3 EMRSA) in Australian hospitals (15).

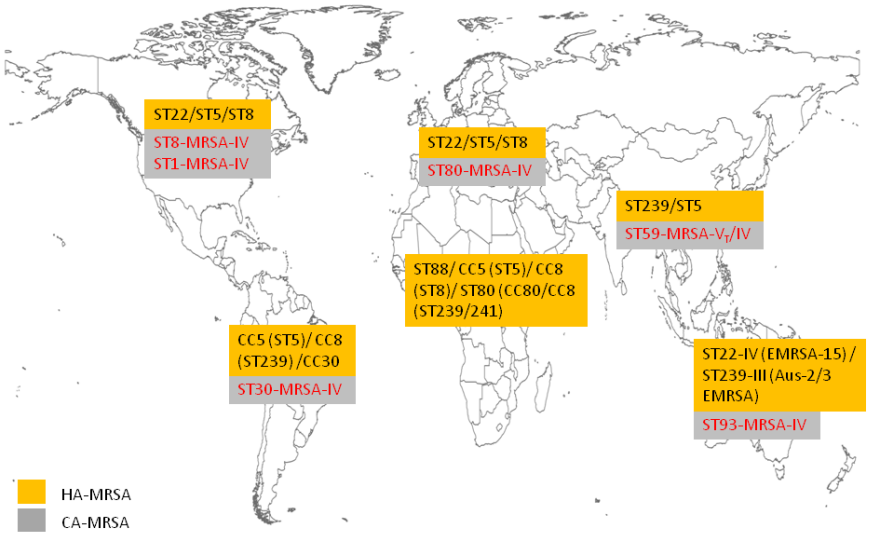

Figure 2. Global distribution of dominant hospital-associated methicillin-resistant *S. aureus* (HA-MRSA) and community associated methicillin-resistant *S. aureus* (CA-MRSA) clones.

CA-MRSA

CA-MRSA was recognized as a distinct entity for a couple of distinguishing features, such as i) a common association with *pvl* genes, ii) a non-multidrug resistant phenotype, and iii) no association with previous exposure to health care. CA-MRSA has now established itself as a major cause of infections in the community as well as in healthcare facilities worldwide. The burden of infections caused by CA-MRSA is increasing among different patient populations globally.

The prevalence of CA-MRSA varies substantially worldwide and the rate of MRSA in all community-associated *S. aureus* infections ranges from less than 1% to more than 50% in different countries (19). High prevalence of CA-MRSA was mainly reported in North America. In a meta-analysis study, the fraction of CA-MRSA out of all *S. aureus* infections was estimated to be 65% in the USA (23). More than 56% of the *Staphylococcus* isolates were CA-MRSA in Northwest Ontario in 2011; this was up from 38% in 2008 (70). Compared to North America, the prevalence of CA-MRSA is much lower in Europe and Asia. However, an increasing trend was observed in some European countries, especially where the incidence

of HA-MRSA is low, such as Denmark (29.4%) or the Netherlands (>21%) (54,105). To date, CA-MRSA is most prevalent in Greece, where PVL-positive strains have emerged as a widespread cause of endemic hospital-acquired infections in some hospitals. In a 2011 study, in 17 hospitals in eight countries — South Korea (7 hospitals), Taiwan (3 hospitals), Hong Kong (1 hospital), Thailand (2 hospitals), the Philippines (1 hospital), Vietnam (1 hospital), India (1 hospital), and Sri Lanka (1 hospital)—the proportion of MRSA among all community-associated *S. aureus* infections in Asian countries ranged from 2.5% to 39%, and four countries (the Philippines, Sri Lanka, Taiwan, and Vietnam) had proportions of greater than 30% (94).

Globally, CA-MRSA strains have shown a remarkable diversity in the number of different clones that have been identified. However, an increasing number of CA-MRSA clones have been reported to have attained epidemic distribution in recent years. ST80 is the dominant CA-MRSA in Europe, ST8 (USA300) and ST1 (USA400) in the USA and Canada, ST59 in Asia, ST93 in Australia and ST772 in India. ST30 is found worldwide, including the USA, Europe, Oceania, Japan, Philippines, Singapore, Malaysia, and Hong Kong (14,92,115). Nevertheless, the exchange of clones between countries and continents has been observed, as can be expected from the current reach, volume and speed of travel.

LA-MRSA

From 1970 to 2000, MRSA was rarely isolated from animals. However, after 2004 ST398 MRSA became epidemic in the Netherlands. A study on the prevalence of MRSA in pigs in slaughterhouses confirmed that LA-MRSA is widely spread among the Dutch pigs, 39% being positive (18). In 2008, the European Food Safety Authority (EFSA) reported the proportion of MRSA-positive production pigs was 26.9%, of which 25.5% being ST398 MRSA. In the USA 49% of the tested pigs and 45% of the swine workers carried MRSA, while in Canada, 25% pigs and 20% of the farmers carried MRSA (49,93). ST398 was the dominant MRSA clone in North America and Korea (57). In contrast to studies in Europe and North America, the predominant strain as found in the majority of Asian pigs belonged to ST9 (38,72).

Although the importance of livestock as a source of MRSA with the potential for epidemic spread in human populations is unclear, it has been shown that some strains could be transmitted from animal to humans and further cause infections in humans, such as ST398, ST130, and CC97 (42, 96,102) .

Methicillin-susceptible *Staphylococcus aureus* (MSSA)

MRSA has evolved from MSSA through the transfer of Staphylococcal Cassette Chromosomes *mec* (SCC*mec*) into extant MSSA lineages. Several studies have shown around 50% of the investigated MSSA isolates had a genetic background common to the major MRSA clones (40,74). Several MSSA lineages have been observed worldwide with a genetic background that is different from the major MRSA clones, such as CC7, CC9, CC12, CC15, CC25, CC51/121, and CC101 (10,37,40). Regionally dominant clones have also been described (68). A recent study from the USA showed a predominance of sequence type 8 (ST8) among MSSA, whereas other strain collections revealed genetic heterogeneity (36).

S. aureus **and MRSA in China**

Hospital associated *S. aureus* (HA-SA)

The first case of MRSA was described in China in the 1970s. Before 2000, 20% of *S. aureus* was identified as MRSA in hospitals; after that, the proportion of MRSA increased rapidly, reaching 70% of *S. aureus* being MRSA in 2005. Although the proportion of MRSA declined from 2006 till now, the high prevalence of MRSA has remained a major problem in China (Figure 3A) (67-69,117-118,120). The antimicrobial resistance spectrum of MRSA in Chinese hospitals is broad with higher resistance rates to macrolides, clindamycin, aminoglycosides and quinolones. An overall high antibiotic resistance rate was observed during the last decade (Figure 3B). Compared to MRSA, MSSA showed a high degree of susceptibility to most antimicrobial agents and only high-level resistance to erythromycin and clindamycin (Figure 3C). No vancomycin-resistant isolates were reported both for MRSA and MSSA until now. A low prevalence of linezolid resistance was observed in 2011.

Two predominant MRSA clones were identified circulating in hospitals in China, ST239-MRSA-SCCmec type III and ST5-MRSA-SCCmec type II (13,63,119,121). ST239 was the most common ST accounting for more than 50% of the isolates in all regions of China. ST5 was the second most common ST nationwide, which was found with greatest frequency in Northeast and East China (32.8% and 16.1%, respectively) and with frequencies of below to 10% in other regions. Importantly, ST59 was identified as an emerging MRSA clone in hospitals of some regions in recent years, but it is noteworthy that ST59 has typical CA-MRSA features (119). The sequence types of MSSA isolated from hospital setting were different from those of MRSA isolates. Although more diversity was found in MSSA, several predominant MSSA clones were reported in Beijing and Shanghai. Seven STs, namely ST398, ST59, ST7, ST15, ST1, ST5 and ST188 account for around 60%

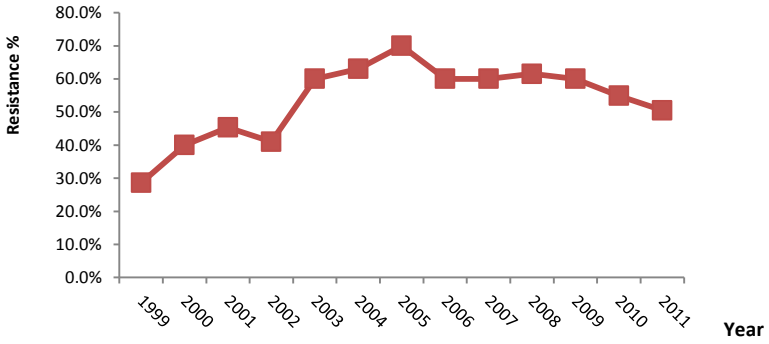

Figure 3A. Trends in the prevalence of MRSA in China from 1999 to 2011.

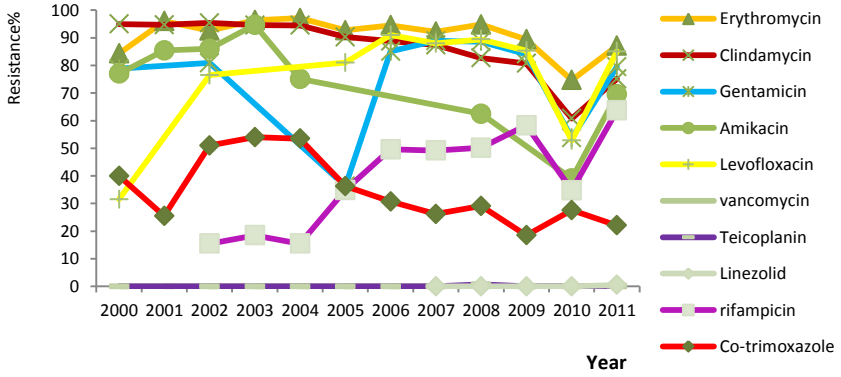

Figure 3B. Antibiotic resistance trends of MRSA in hospitals in China (Data from MOH National Antibacterial Resistance Investigation Net (Mohnarin)).

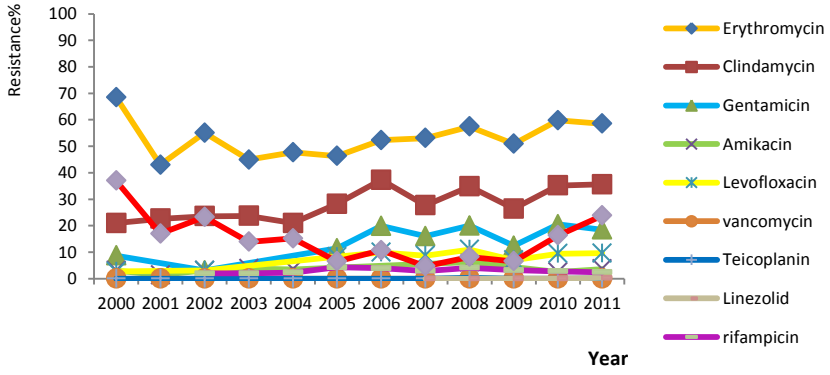

Figure 3C. Antibiotic resistance trends of MSSA in hospitals in China (Data from MOH National Antibacterial Resistance Investigation Net (Mohnarin)).

of the MSSA strains in hospitals (12,95). An unusually high prevalence of PVL-producing ST22-MSSA-t309 strains in a hospital form Urumqi in the Northwest of China (Inner Mongolia) was for the first time reported in a recent study (13). An average prevalence of PVL, ranging from 2% to 11%, was observed in Chinese MRSA strains (13, 43, 63, 119), most of which belonged to the ST59 type. It has been reported that 43% of the MRSA and 10% of the MSSA isolates from blood culture in one hospital in the Zhejiang province were PVL-positive (124).

Community associated *S. aureus* (CA-SA)

The incidence of CA-MRSA in China is unclear because of the lack of systematic epidemiological studies. Most investigations about CA-SA focused on skin and soft tissue infections (SSTIs). The prevalence and ST types of CA-MRSA showed differences in the geographical distribution. The prevalence of CA-MRSA among adults with SSTIs was 0-3% in Beijing (47,129) and 21.3% in Wenzhou (Zhejiang province) (123). CA-MSSA was susceptible to all antibiotics with the exception of high resistance rates to erythromycin, clindamycin, tetracycline and gentamicin. More like HA-MRSA, CA-MRSA was resistant to the majority of antibiotics except of vancomycin, teicoplanin, linezolid, daptomycin, and tigecycline (47,129).

ST1018, ST239 and ST88 mainly found amongst CA-SA of which the ST1018-MRSA-SCC*mec* III clone was more likely to be obtained in Wenzhou (123). In Beijing, six major MSSA clones (ST398, ST7, ST1, ST59, and ST5) were responsible for SSTIs of which ST398 was the most prevalent clone with high prevalence of PVL (64.3%). Four MRSA types (ST239, ST59, ST8, and ST6) were found in Beijing as well.

The incidence of CA-MRSA in children with skin and soft tissue infections was 4% in Beijing (116). A notable increase in invasive CA-MRSA infections from 0% to 2.43% over 6 years time was observed in one Beijing hospital (81). Compared with CA-MSSA, the resistance rates of CA-MRSA to ciprofloxacin, chloramphenicol, gentamicin and tetracycline were higher (110). CA-MRSA isolates were found to be associated largely with the ST59 MRSA-IV clone, mostly causing SSTIs (42.9%-58.6%) and pneumonia (40.4%). Of all isolates mentioned in these studies, 71.4% of CA-MRSA and 4.17% of MSSA isolates harbored *pvl* (30,31,116). ST398 MRSA strains were only occasionally reported both in adults and in children (30,123). Unlike MRSA, no predominant *spa* type was seen in MSSA strains. In one hospital of Beijing, the most common *spa* types among MSSA consisted of t084 (8.3%), t091 (5.8%), t034 (5%), t127 (4.2%), t002 (4.2%), and t796 (4.2%) (116).

To date, there have been only few reports on the prevalence and the risk factors of *S. aureus*

nasal colonisation in China. Previous studies revealed 15.4-23.1% *S. aureus* nasal carriage in Chinese medical students from different regions, of which 3.0-9.4% were MRSA (22,65). Another study revealed a similar nasal carriage rate (20%) in 1,044 military volunteers from Beijing whereby no MRSA strains were identified (82).

Livestock associated *S. aureus* (LA-SA)

Although there was a paucity of data regarding characteristics of *S. aureus*, especially MSSA, in food animals in China, several conclusions still can be drawn from the published studies (11, 17, 44, 108, 112, 128): 1) A high prevalence of MRSA was found among pigs. 2) ST9, *spa* type t899 and SCC*mec* III/IVb was the main MRSA clone from porcine origin. 3) More diversity was found among pig MSSA isolates than among pig MRSA isolates. ST9 and ST15 were the major MSSA clones. 4) There was a higher prevalence of antimicrobial resistance in pig isolates than in cattle isolates, with high resistance rates to erythromycin (95.2-100%), ciprofloxacin (92.8%) and gentamicin (67.5%) (112,127). 5) A recent study, reported a high incidence of oxacillin-susceptible *mecA*-positive *S. aureus* (OS-MRSA) in bovine mastitis in China with isolates belonging to different clones (80).

Evolution of *S. aureus* as a species

S. aureus evolves mainly due to the accumulation of point mutations and selection, and to a minor degree due to the horizontal gene transfer (HGT) of mobile genetic elements (MGEs) originating from the same (intra-species) or different species (inter-species). Thus, the population of *S. aureus* presents a highly clonal structure. It has been shown that the majority of *S. aureus* isolates, including both human carriage and clinical isolates, can be grouped into the 11 phylogenetic lineages or clonal complexes, whereas most MRSA isolates belong to five clonal complexes. Isolates from the same clonal complex have remarkably conserved genomes (except for MGEs). Examination of the sequence changes at multilocus sequence typing (MLST) loci has shown that point mutations give rise to new alleles at least 15-fold more frequently than does recombination (27). In contrast, alleles change between 5- and 10-fold more frequently by recombination than by mutation in naturally transformable species, such as *Neisseria meningitidis* and *Streptococcus pneumoniae*. Isolates from the same clonal complex have a consistent set of genes in common, which vary between clonal complexes and are referred to as “core variable” (CV) genes. The majority of the CV genes are associated with surface proteins, which are known to directly interact with host components (61). In the absence of homologous recombination mediated by HGT, adaptation in the core genome would be limited by the supply of new mutations to adapt to new selection pressures: resistance to many antibiotics is conferred by substitutions in highly conserved

core genes (35).

Except for rare chromosomal replacements (85), there is also little genetic exchange between clonal complexes in *S. aureus*, thus maintaining the clonal evolutionary structure of the species as a whole. The probable reason for this is that random transfer of DNA is restricted in *S. aureus.* In the laboratory, *S. aureus* is difficult to be modified genetically, as evidenced by the rejection of exogenous plasmids. In addition, each lineage of *S. aureus* has its own phage pattern and the previously established phage typing is based on differences in the specific phage pattern of each complex.

Restriction–modification (RM) systems are responsible for protecting the host from invasion by foreign DNA. Up to now, three different RM systems (type I, type II and type IV) have been described for *S. aureus.* The type I RM system is considered to contribute to the evolution of the different lineages. This RM system is found in nearly all isolates of *S. aureus* where it is located in the genomic islands, γSaα and γSaβ, respectively. A type I RM system contains three genes, one copy of *hsdR* (for restriction), two different copies of *hsdM* (for modification), and two different copies of *hsdS* (for specificity) at distant locations (71,109). The *hsdS* genes are highly conserved among isolates from the same clonal complex (CC), but vary between complexes. Therefore, strains from the same complex can exchange DNA at a higher frequency than strains from different complexes (84,109). This mechanism explains the evolution and differentiation of *S. aureus* clonal complexes. Type II restriction enzymes were the first RM system to be characterized in *S. aureus*. This system is widely used in genetic laboratories, but uncommon in typical *S. aureus* isolates(89). In the type IV RM system found in *S. aureus*, only the restriction enzyme is present, which is responsible for recognition and digestion of introduced DNA from different species (122). It has been shown that some clonal complexes may be deficient in certain restriction functions, which explains the occasional acquisition of antibiotic resistance determinants from different species such as *vanA* from enterococci (16).

Although recombination occurs at a low frequency in *S. aureus*, its significance in the evolution of this species should not be underestimated. Sometimes, recombination can even change the relationships between STs. Phylogenetic analysis suggests that homologous recombination does contribute toward the evolution of this species over the long term. It has been shown that recombination will affect the *arcC* locus, one of the seven housekeeping loci for MLST analysis, and may influence its sequence evolution (27). Large chromosomal replacements have been identified in *S. aureus,* although rarely occurring naturally. The well-known pandemic lineage of MRSA, ST239, is known to have emerged through a very

large (635 kb) homologous replacement from ST30 to an ST8 parent via an unknown mechanism (85).

During the evolution of *S. aureus*, new epidemic clones arise and old ones disappear. Although the real mechanism of this phenomenon is still unclear, it is becoming clearer that MGEs are an important driver of the evolution of new clones that adapt to new niches (59). MGEs encode many virulence, immune evasion and antibiotic resistance genes, and they can transfer at high frequency between isolates of the same lineage by horizontal gene transfer (HGT). A novel surface protein named SasX, which is always linked to a novel prophage was recently suggested as the driving force of the Asian MRSA epidemic (55). In a recent study, a series of mobile element-driven recombination hotspots in the core genome were identified, which include regions flanking the conjugative transposon ICE6013, SCC and genomic island γSaα, which represents an opportunity for adaptation and challenges our understanding of the recombination landscape (24).

Molecular typing methods

Because *S. aureus* is a highly clonal species, polymorphisms in coding or noncoding regions on the chromosome will allow a largely congruent classification of isolates into different clonal complexes. With the improvement of recent molecular technology, more and more molecular typing methods were developed to replace traditional phenotype typing. The ideal requirements for typing techniques have been defined with the following characteristics (103): 1) total typeability; 2) excellent discriminatory ability; 3) unambiguous, highly reproducible and portable data; 4) intra/inter-laboratory comparability; 5) speed (<3 days) and low-cost; 6) ease of performance, interpretation, storage and exchange of data; 7) international standardized nomenclature; and 8) epidemiological concordance. However up to now, no single method can fulfill all these criteria. The main available *S. aureus* molecular typing methods are summarized in Table 2. The choice of an appropriate molecular typing method (or methods) depends crucially on the study question, epidemiological background, time and geographical resolution that is required.

For outbreak investigations, highly discriminatory methods have been recommended, such as pulsed-field gel electrophoresis (PFGE), multiple-locus variable number tandem repeat (VNTR) analysis/fingerprinting (MLVA/MLVF), *spa* typing, MLST and whole-genome sequencing (WGS). There is a trend of PFGE being replaced by MLVA. Improved MLVA methods have been developed for *S. aureus* in recent years, which provide not only a high discriminative ability but also allow for phylogenetic conclusions (79,88). *Spa* typing is an easy, cheap and rapid typing method with versatile clinical utility. Hospital laboratory staff can

create their own dabatase based on *spa* typing results. In recent year, several studies of MRSA outbreaks in hospitals using WGS were reported (25, 41,51). This work identified sufficient variation between *S. aureus* genomes to be able to describe the spread of bacteria between patients during an outbreak.

At the regional (within-country) level, an understanding of the distribution of strains between different hospitals complements the micro-epidemiology investigation at single institutions. For this purpose, MLST and *spa* typing would provide sufficient detail. For cost reasons *spa* typing has become one of the primary typing methods for regional and national MRSA surveillance schemes (29,97). MLST is based on allelic polymorphism in seven neutral housekeeping genes which is an extremely useful method to define the core genetic population structure of *S. aureus,* and is suitable for long-term evolution studies.

Genomic characterization of *S. aureus*

The first complete genomes of *S. aureus* were sequenced in 2001 (52). Since then, at least 52 completely sequenced genomes have become available in the public database together wiht thousands of draft or incompletely assembled genomes. Most *S. aureus* genomes are approximately 2.8 Mbp in size and the genomes of all sequenced strains have a very similar architecture. The *S. aureus* genome is comprised of a highly conserved gene structure present in all strains, designated the “core” genome (60), and an accessory genome that is variable across all the strains. The core genome is around 2.3 Mbp in size and contains housekeeping genes, genes required for metabolic functions and a number of conserved virulence genes. MGEs account for 15%-20% of genome and mainly include bacteriophages, pathogenicity islands (SaPI), plasmids, transposons and the SCC*mec*.

The three families of genomic islands, νSaα, νSaβ and νSaγ, are located on the chromosome and have been found in nearly all *S. aureus* strains (28,33). νSaαencodes for a cluster of staphylococcal superantigen-like proteins, the so-called *set* cluster, and a cluster of lipoproteins (*lpl* cluster), while νSaβ encodes for a serine protease cluster (*spl* cluster) and an enterotoxin cluster. The νSaγ genomic islands contain genes encoding for phenol-soluble modulins (PSMs) and a cluster of new types of enterotoxin-like toxin (SEI) genes similar to the one described within νSaα.

Table 2. Comparison of the typing methods for *S. aureus*

Methods	Principle	Advantage	Limitation
Random Amplification of Polymorphic DNA(RAPD)	Unspecific binding with polymorphism of the whole chromosome	Simple, inexpensive, rapid and easy in use	Less discriminatory, low intra/inter-laboratory reproducibility
Repetitive sequence-based PCR (Rep-PCR)	Polymorphism of noncoding intergenic repetitive sequences of the genome	Inexpensive, fast	Low reproducibility
DiversiLab (semiautomated Rep-PCR)	Polymorphism of noncoding intergenic repetitive sequences of the genome	Easy to perform, fast, reproducible	Expensive, low discriminatory power, limited portability, subjectivity of results analysis
Pulsed-field gel electrophoresis (PFGE)	Restriction polymorphism of the whole chromosome	Highly discriminatory, high epidemiological concordance, excellent intra-laboratory reproducibility	Technically demanding, slow, subjectivity of results analysis, limited interlaboratory portability, multiple nomenclatures
spa typing	Sequence polymorphism in the variable X region of the *spa* gene	Cheap, rapid, easy to perform, high throughput, portability, standard nomenclature	Misclassification of a small number of lineages
SCC*mec* typing	structure of SCC*mec* region	Distinguishes different SCC*mec* types	Only relevant for MRSA

Mutilocus sequence typing (MLST)	Sequence determination of allelic variants of housekeeping genes	Portability, standard nomenclature	Limited discriminatory power, low throughput, high cost
Multilocus variable-number tandem repeat (VNTR) analysis (MLVA)	Polymorphism in chromosomal VNTR elements	Relatively cheap, rapid, high throughput, very good discriminatory power, interlaboratory portability	No validated interpretation criteria, no standard nomenclature, misclassification of some lineages
Multiple locus VNTR fingerprinting(MLVF)	Polymorphism in chromosomal VNTR elements	Simple, very cheap, rapid, high throughput, easy to perform, excellent discriminatory power	Limited portability, subjectivity of results analysis, no standard nomenclature
Genome-scale DNA microarrays	Hybridization with genes on the chromosome and plasmids	High resolution	Expensive, slow, technically demanding
Optical mapping	Whole-genome mapping	High resolution	Extremely expensive
Genome sequencing	Genome-wide variations	High resolution, fast, became recently cheaper, large amounts of information	Technically demanding, bioinformatics analysis demanding
Matrix-Assisted Laser Desorption Ionization - Time of Flight (MALDI-TOF) Mass Spectrometry (MS)	Generation of massspectral fingerprints from bacterial whole cells or crude extracts	Fast, high resolution, less labor	Technically demanding, equipment demanding, relatively low inter-lab reproducibility, not yet sufficiently validated, distinguishes isolates only at the species level

The staphylococcal cassette chromosome *mec* (SCC*mec*) is a relatively large DNA fragment on the chromosome that always integrates at a specific site (*attB* or the integration site sequence ISS) within the 3' end of the *orfX* gene encoding a ribosomal methyltransferase (8). Different SCC*mec* elements share a similar backbone structure, that consists of (i) the *mec* complex, composed of the *mecA* operon, (ii) the *ccr* gene complex, composed of cassette chromosome recombinase (*ccr*) gene(s), and (iii) three regions bordering the *ccr* and *mec* complexes, designated as joining (J) regions. Four classes of the *mec* complex and seven *ccr* complexes have been described to date in MRSA (http://www.sccmec.org). According to the combination of different classes of *mec* and *ccr* gene complexes, eleven SCC*mec* types have been reported in *S. aureus,* ranging in size from 20 to 60 kb (91). Many different SCC*mec* subtypes have also been described that harbour the same *ccr* and *mec* gene combination, but vary in the J regions. Atypical SCC/SCC*mec* elements including SCC elements (harbouring a *ccr* complex but lacking *mecA*), Pseudo (Ψ) SCC*mec* elements (carrying *mecA* but lacking the *ccr* complex) and Pseudo (Ψ) SCC elements (lacking *ccr* and *mec* genes) were reported recently.

All known *S. aureus* phages belong to the order of Caudovirales (tailed phages). Based on the tail morphology, they are further classified into three major families: Podoviridae, Siphoviridae, and Myoviridae. The genome sizes of staphylococcal phages extend from less than 16kb to more than 140kb, and they are classified into three size classes: class I, podoviruses (<16-20kb); class II, siphoviridae (39-43kb); and class III, myoviruses, (120-140kb) (53). Genomes of the Siphoviridae are usually organized into six functional modules, known as the lysogeny, DNA replication, packaging, head, tail, and lysis modules. Phage genomes of different serogroups share the highest nucleotide sequence similarity at the replication site. Multiple alignments of *S. aureus* phage genomes reveal a chimeric and mosaic structure resulting from horizontal gene transfer and recombination (67). For this reason, it was suggested to classify the prophages of *S. aureus* on the basis of *int* gene homology (34). In *S. aureus,* several phage-borne genes for virulence factors have been described, such as *sea* (for enterotoxin A), PV-*luk* (for Panton-Valentine leukocidin), *scin* (for complement inhibitor protein), *chip* (for chemotaxis inhibitory protein), *sak* (for staphylokinase), *eta* (for exfoliative toxin A), and *sasX* (for the surface protein SasX) (55,58). These accessory phage genes enable *S. aureus* lineages to estabilish themselves in different reservoirs and are thus major determinants for *S. aureus* evolution.

The SaPIs are 12 to 27kb mobile pathogenicity islands encoding integrase, resistance, and virulence genes. Each *S. aureus* genome contains one or more SaPIs. These SaPIs rely on

helper phages for horizontal transfer, because they lack the genes necessary for constructing capsid heads and tails for gene transfer (66). To date, more than 23 prototypes of SaPIs have been sequenced, all of which are inserted into the chromosome at specific sites (1). SaPIs can carry genes for toxic shock syndrome toxin-1(*tst*), superantigens (*seb, sec, sek, seq, sel*), and other pathogenicity factors (*ear, eta, bap*).

The majority of natural *S. aureus* isolates contain one or more plasmids of 1-60kbp. The plasmids in *S. aureus* were classified into three different types. The class I plasmids are small rolling circle plasmids (1-5kb) with only one or two resistance determinants (e.g. pT181 and pC194). The class II plasmids are larger (15-46kb) and replicate by the theta mechanism with lower copy numbers. This group includes penicillinase and aminoglycoside/trimethoprim resistance plasmids (e.g. pSK 1 and pIP630). The class III plasmids consist of large (30-60kbp) plasmids that encode the *tra* genes for conjugation and always carry a combination of resistance markers (1,58).

Scope of this thesis

The research presented in this thesis focuses on the genetic population structure of *S. aureus* isolated from clinical specimen and healthy carriers. In addition, it addresses isolates from pigs and episodes of food poisoning. Importantly, all investigated isolates were collected in China and they were compared to a representative European strain collection. To better understand the genomic structure of Chinese porcine *S. aureus* isolates that carry a novel mobile genetic element conveying antibiotic resistance, whole-genome sequencing was applied. The different aspects related to this research are introduced in **Chapter 1.**

Chapter 2 describes the dynamics of MRSA over a 5-year period (from 2000 to 2005) at a representative hospital in Beijing, China. This study revealed increasing levels of antimicrobial resistance and epidemiological changes in the hospital-associated MRSA strains isolated in this facility.

Chapter 3 provides a first description of isolates of *S*. a*ureus* that had caused staphylococcal food poisoning (SFP) in Southern China. The two dominant *S. aureus* lineages, (i) PFGE types A and B with ST6 and (ii) PFGE type C with ST943, were identified during outbreaks.

Chapter 4 focuses on the prevalence and genetic population structure of *S. aureus* isolates that colonize pigs in North-Eastern China. A novel finding is that *S. aureus* ST398 is indeed a frequent colonizer of pigs but, in contrast to the European porcine isolates where MRSA dominates, the Chinese porcine isolates are mostly shown to display a multiresistant MSSA phenotype. Notably, a novel gene that was recently described to confer quinupristin/dafloprístin resistance was very frequently identified in the Chinese porcine *S. aureus* isolates. This is probably a consequence of the use of virginiamycin which, in contrast to Europe and the USA, is not banned in China.

Chapter 5 reports on the risk factors for *S. aureus* colonization and those associated with specific clonal lineages expressing certain genetic traits in healthy carriage in Northern China. Our study showed that younger people(≤24 years) and ethnic non-Han individuals were more likely colonized with *S. aureus*. Furthermore, the presence of household members who are healthcare personnel appeared to be a risk factor for MC398 carriage. Higher frequency of both *S. aureus* carriage and MC398 carriage was found in Harbin.

Chapter 6 reports on the first attempt to describe two extant populations of *S. aureus* isolated during dedicated surveys from opposite ends of the same continental shelf - China and Europe. The results show that there is a systematic difference in the distribution of *S. aureus* clonal lineages between Europe and China. On this basis, it can be argued that the relative

frequencies of Chinese vs. European isolates among founders and descendants point to different geographic origins of several lineages.

Chapter 7 describes investigations on the genetic backgrounds of the recently identified *lsa*(*E*)-positive porcine *S. aureus* ST9 isolates from China, which were described in **chapter 4** by whole-genome sequencing (WGS). Phylogenetic analyses show that the sequenced isolates belong to a distinct evolutionary cluster closely related to clonal complex 5 (CC5). Further analysis revealed that all isolates were deficient in the recently described type IV restriction-modification system. A novel type V (5C2)-like SCC*mec* element was identified in the two MRSA strains, which included a class C2 *mec* gene complex and a new allotype *ccrC* gene. Our WGS analysis indicate that the sequenced quinupristin/dalfopristin resistant ST9 lineage represents a reservoir of potentially new mobile genetic elements associated with new resistance features that may have originated from other species and spread further to successful *S. aureus* lineages.

Chapter 8 addresses the novel *S. aureus* surface protein SasX, which was recently associated with epidemic MRSA clones in China. The results show that SasX can slightly antagonize the colony spreading motility of *S. aureus* as assayed on a soft agar medium.

Finally, **chapter 9** provides an overall discussion and suggests avenues for future research.

Reference List

1. Alibayov, B., L. Baba-Moussa, H. Sina, K. Zdenkova, and K. Demnerova. 2014. *Staphylococcus aureus* mobile genetic elements. Mol.Biol.Rep. 41:5005-18
2. Arianpoor, A., E. Askari, F. Soleymani, S. M. Tabatabai, M. Rahbar, and N. M. Naderi. 2012. Updates on the global prevalence of MRSA: results from Iran. Int.J.Antimicrob.Agents 40:373.
3. Armand-Lefevre, L., R. Ruimy, and A. Andremont. 2005. Clonal comparison of *Staphylococcus aureus* isolates from healthy pig farmers, human controls, and pigs. Emerg.Infect.Dis. 11:711-4.
4. Aubry-Damon, H., P. Legrand, C. Brun-Buisson, A. Astier, C. J. Soussy, and R. Leclercq. 1997. Reemergence of gentamicin-susceptible strains of methicillin-resistant *Staphylococcus aureus*: roles of an infection control program and changes in aminoglycoside use. Clin.Infect.Dis. 25:647-53.
5. Beck, W. D., B. Berger-Bachi, and F. H. Kayser. 1986. Additional DNA in methicillin-resistant *Staphylococcus aureus* and molecular cloning of *mec*-specific DNA. J.Bacteriol. 165:373-8.
6. Berube, B. J. and W. J. Bubeck. 2013. *Staphylococcus aureus* alpha-toxin: nearly a century of intrigue. Toxins.(Basel) 5:1140-66.
7. Boone DR, Castenholz RW, Garrity GM, and Bergey DH. 2001. Bergey's manual of systematic bacteriology. Springer,New York.
8. Boundy, S., M. K. Safo, L. Wang, F. N. Musayev, H. C. O'Farrell, J. P. Rife, and G. L. Archer. 2013. Characterization of the *Staphylococcus aureus* rRNA methyltransferase encoded by *orfX*, the gene containing the staphylococcal chromosome Cassette *mec* (SCC*mec*) insertion site. J.Biol.Chem. 288:132-40.
9. Bradley, A. 2002. Bovine mastitis: an evolving disease. Vet.J. 164:116-28.
10. Chambers, H. F. and F. R. Deleo. 2009. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. Nat.Rev.Microbiol. 7:629-41.
11. Chao, G., G. Bao, and X. Jiao. 2014. Molecular Epidemiological Characteristics and Clonal Genetic Diversity of *Staphylococcus aureus* with Different Origins in China. Foodborne.Pathog.Dis.11:503-10.
12. Chen, H., Y. Liu, X. Jiang, M. Chen, and H. Wang. 2010. Rapid change of methicillin-resistant *Staphylococcus aureus* clones in a Chinese tertiary care hospital over a 15-year period. Antimicrob.Agents Chemother. 54:1842-7.
13. Chen, Y., Z. Liu, L. Duo, J. Xiong, Y. Gong, J. Yang, Z. Wang, X. Wu, Z. Lu, X. Meng, J. Zhao, C. Zhang, F. Wang, Y. Zhang, M. Zhang, and L. Han. 2014. Characterization of *Staphylococcus aureus* from Distinct Geographic Locations in China: An Increasing Prevalence of *spa*-t030 and SCC*mec* Type III. PLoS One 9:e96255.
14. Chuang, Y. Y. and Y. C. Huang. 2013. Molecular epidemiology of community-associated meticillin-resistant *Staphylococcus aureus* in Asia. Lancet Infect.Dis. 13:698-708.
15. Coombs, G. W., G. R. Nimmo, J. C. Pearson, P. J. Collignon, J. M. Bell, M. L. McLaws, K. J. Christiansen, and J. D. Turnidge. 2013. Australian Group on Antimicrobial Resistance Hospital-onset *Staphylococcus aureus* Surveillance Programme annual report, 2011. Commun.Dis.Intell.Q.Rep. 37:E210-8.
16. Corvaglia, A. R., P. Francois, D. Hernandez, K. Perron, P. Linder, and J. Schrenzel. 2010. A type III-like restriction endonuclease functions as a major barrier to horizontal gene transfer in clinical *Staphylococcus aureus* strains. Proc.Natl.Acad.Sci.U.S.A 107:11954-8.
17. Cui, S., J. Li, C. Hu, S. Jin, F. Li, Y. Guo, L. Ran, and Y. Ma. 2009. Isolation and characterization of methicillin-resistant *Staphylococcus aureus* from swine and workers in China. J.Antimicrob.Chemother. 64:680-3.

18. de Neeling, A. J., M. J. van den Broek, E. C. Spalburg, M. G. van Santen-Verheuvel, W. D. Dam-Deisz, H. C. Boshuizen, A. W. van de Giessen, D. E. van, and X. W. Huijsdens. 2007. High prevalence of methicillin resistant *Staphylococcus aureus* in pigs. Vet.Microbiol. 122:366-72.
19. Deurenberg, R. H. and E. E. Stobberingh. 2008. The evolution of *Staphylococcus aureus*. Infect.Genet.Evol. 8:747-63.
20. Devriese, L. A., L. R. Van Damme, and L. Fameree. 1972. Methicillin (cloxacillin)-resistant *Staphylococcus aureus* strains isolated from bovine mastitis cases. Zentralbl.Veterinarmed.B 19:598-605.
21. Draghi, D. C., D. J. Sheehan, P. Hogan, and D. F. Sahm. 2005. In vitro activity of linezolid against key gram-positive organisms isolated in the united states: results of the LEADER 2004 surveillance program. Antimicrob.Agents Chemother. 49:5024-32.
22. Du, J., C. Chen, B. Ding, J. Tu, Z. Qin, C. Parsons, C. Salgado, Q. Cai, Y. Song, Q. Bao, L. Zhang, J. Pan, L. Wang, and F. Yu. 2011. Molecular characterization and antimicrobial susceptibility of nasal *Staphylococcus aureus* isolates from a Chinese medical college campus. PLoS One 6:e27328.
23. Dukic, V. M., D. S. Lauderdale, J. Wilder, R. S. Daum, and M. Z. David. 2013. Epidemics of community-associated methicillin-resistant *Staphylococcus aureus* in the United States: a meta-analysis. PLoS One 8:e52722.
24. Everitt, R. G., X. Didelot, E. M. Batty, R. R. Miller, K. Knox, B. C. Young, R. Bowden, A. Auton, A. Votintseva, H. Larner-Svensson, J. Charlesworth, T. Golubchik, C. L. Ip, H. Godwin, R. Fung, T. E. Peto, A. S. Walker, D. W. Crook, and D. J. Wilson. 2014. Mobile elements drive recombination hotspots in the core genome of *Staphylococcus aureus*. Nat.Commun. 5:3956.
25. Eyre, D. W., T. Golubchik, N. C. Gordon, R. Bowden, P. Piazza, E. M. Batty, C. L. Ip, D. J. Wilson, X. Didelot, L. O'Connor, R. Lay, D. Buck, A. M. Kearns, A. Shaw, J. Paul, M. H. Wilcox, P. J. Donnelly, T. E. Peto, A. S. Walker, and D. W. Crook. 2012. A pilot study of rapid benchtop sequencing of *Staphylococcus aureus* and *Clostridium difficile* for outbreak detection and surveillance. BMJ Open. 2. e001124.
26. Falagas, M. E., D. E. Karageorgopoulos, J. Leptidis, and I. P. Korbila. 2013. MRSA in Africa: filling the global map of antimicrobial resistance. PLoS One 8:e68024.
27. Feil, E. J., J. E. Cooper, H. Grundmann, D. A. Robinson, M. C. Enright, T. Berendt, S. J. Peacock, J. M. Smith, M. Murphy, B. G. Spratt, C. E. Moore, and N. P. Day. 2003. How clonal is *Staphylococcus aureus*? J.Bacteriol. 185:3307-16.
28. Fitzgerald, J. R., S. D. Reid, E. Ruotsalainen, T. J. Tripp, M. Liu, R. Cole, P. Kuusela, P. M. Schlievert, A. Jarvinen, and J. M. Musser. 2003. Genome diversification in *Staphylococcus aureus*: Molecular evolution of a highly variable chromosomal region encoding the Staphylococcal exotoxin-like family of proteins. Infect.Immun. 71:2827-38.
29. Friedrich, A. W., W. Witte, L. H. de, W. Hryniewicz, J. Scheres, and H. Westh. 2008. A European laboratory network for sequence-based typing of methicillin-resistant *Staphylococcus aureus* (MRSA) as a communication platform between human and veterinary medicine--an update on SeqNet.org. Euro.Surveill 13.
30. Geng, W., Y. Yang, D. Wu, G. Huang, C. Wang, L. Deng, Y. Zheng, Z. Fu, C. Li, Y. Shang, C. Zhao, S. Yu, and X. Shen. 2010. Molecular characteristics of community-acquired, methicillin-resistant *Staphylococcus aureus* isolated from Chinese children. FEMS Immunol.Med.Microbiol. 58:356-62.
31. Geng, W., Y. Yang, D. Wu, W. Zhang, C. Wang, Y. Shang, Y. Zheng, L. Deng, Z. Fu, X. Li, S. Yu, and X. Shen. 2010. Community-acquired, methicillin-resistant

Staphylococcus aureus isolated from children with community-onset pneumonia in China. Pediatr.Pulmonol. 45:387-94.

32. Ghaznavi-Rad, E., S. M. Nor, Z. Sekawi, L. Y. Khoon, M. N. Aziz, R. A. Hamat, N. Othman, P. P. Chong, B. A. van, H. Ghasemzadeh-Moghaddam, and V. Neela. 2010. Predominance and emergence of clones of hospital-acquired methicillin-resistant *Staphylococcus aureus* in Malaysia. J.Clin.Microbiol. 48:867-72.
33. Gill, S. R., D. E. Fouts, G. L. Archer, E. F. Mongodin, R. T. Deboy, J. Ravel, I. T. Paulsen, J. F. Kolonay, L. Brinkac, M. Beanan, R. J. Dodson, S. C. Daugherty, R. Madupu, S. V. Angiuoli, A. S. Durkin, D. H. Haft, J. Vamathevan, H. Khouri, T. Utterback, C. Lee, G. Dimitrov, L. Jiang, H. Qin, J. Weidman, K. Tran, K. Kang, I. R. Hance, K. E. Nelson, and C. M. Fraser. 2005. Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. J.Bacteriol. 187:2426-38.
34. Goerke, C., R. Pantucek, S. Holtfreter, B. Schulte, M. Zink, D. Grumann, B. M. Broker, J. Doskar, and C. Wolz. 2009. Diversity of prophages in dominant *Staphylococcus aureus* clonal lineages. J.Bacteriol. 191:3462-8.
35. Gordon, N. C., J. R. Price, K. Cole, R. Everitt, M. Morgan, J. Finney, A. M. Kearns, B. Pichon, B. Young, D. J. Wilson, M. J. Llewelyn, J. Paul, T. E. Peto, D. W. Crook, A. S. Walker, and T. Golubchik. 2014. Prediction of *Staphylococcus aureus* antimicrobial resistance by whole-genome sequencing. J.Clin.Microbiol. 52:1182-91.
36. Grundmann, H., D. M. Aanensen, C. C. van den Wijngaard, B. G. Spratt, D. Harmsen, and A. W. Friedrich. 2010. Geographic distribution of *Staphylococcus aureus* causing invasive infections in Europe: a molecular-epidemiological analysis. PLoS Med. 7:e1000215.
37. Grundmann, H., S. Hori, M. C. Enright, C. Webster, A. Tami, E. J. Feil, and T. Pitt. 2002. Determining the genetic structure of the natural population of *Staphylococcus aureus*: a comparison of multilocus sequence typing with pulsed-field gel electrophoresis, randomly amplified polymorphic DNA analysis, and phage typing. J.Clin.Microbiol. 40:4544-6.
38. Guardabassi, L., M. O'Donoghue, A. Moodley, J. Ho, and M. Boost. 2009. Novel lineage of methicillin-resistant *Staphylococcus aureus*, Hong Kong. Emerg.Infect.Dis. 15:1998-2000.
39. Guzman-Blanco, M., C. Mejia, R. Isturiz, C. Alvarez, L. Bavestrello, E. Gotuzzo, J. Labarca, C. M. Luna, E. Rodriguez-Noriega, M. J. Salles, J. Zurita, and C. Seas. 2009. Epidemiology of methicillin-resistant *Staphylococcus aureus* (MRSA) in Latin America. Int.J.Antimicrob.Agents 34:304-8.
40. Hallin, M., O. Denis, A. Deplano, M. R. De, R. R. De, S. Rottiers, and M. J. Struelens. 2007. Genetic relatedness between methicillin-susceptible and methicillin-resistant *Staphylococcus aureus*: results of a national survey. J.Antimicrob.Chemother. 59:465-72.
41. Harris, S. R., E. J. Cartwright, M. E. Torok, M. T. Holden, N. M. Brown, A. L. Ogilvy-Stuart, M. J. Ellington, M. A. Quail, S. D. Bentley, J. Parkhill, and S. J. Peacock. 2013. Whole-genome sequencing for analysis of an outbreak of meticillin-resistant *Staphylococcus aureus*: a descriptive study. Lancet Infect.Dis. 13:130-6.
42. Harrison, E. M., G. K. Paterson, M. T. Holden, J. Larsen, M. Stegger, A. R. Larsen, A. Petersen, R. L. Skov, J. M. Christensen, Z. A. Bak, O. Heltberg, S. R. Harris, R. N. Zadoks, J. Parkhill, S. J. Peacock, and M. A. Holmes. 2013. Whole genome sequencing identifies zoonotic transmission of MRSA isolates with the novel *mecA* homologue *mecC*. EMBO Mol.Med. 5:509-15.

43. He, W., H. Chen, C. Zhao, F. Zhang, H. Li, Q. Wang, X. Wang, and H. Wang. 2013. Population structure and characterisation of *Staphylococcus aureus* from bacteraemia at multiple hospitals in China: association between antimicrobial resistance, toxin genes and genotypes. Int.J.Antimicrob.Agents 42:211-9.
44. He, W., Y. Liu, J. Qi, H. Chen, C. Zhao, F. Zhang, H. Li, and H. Wang. 2013. Food-animal related *Staphylococcus aureus* multidrug-resistant ST9 strains with toxin genes. Foodborne.Pathog.Dis. 10:782-8.
45. Herman, A., N. Labrecque, J. Thibodeau, P. Marrack, J. W. Kappler, and R. P. Sekaly. 1991. Identification of the staphylococcal enterotoxin A superantigen binding site in the beta 1 domain of the human histocompatibility antigen HLA-DR. Proc.Natl.Acad.Sci.U.S.A 88:9954-8.
46. Hudson, L. O., C. Reynolds, B. G. Spratt, M. C. Enright, V. Quan, D. Kim, P. Hannah, L. Mikhail, R. Alexander, D. F. Moore, D. Godoy, C. J. Bishop, and S. S. Huang. 2013. Diversity of methicillin-resistant *Staphylococcus aureus* strains isolated from residents of 26 nursing homes in Orange County, California. J.Clin.Microbiol. 51:3788-95.
47. Jiang, W., Z. Zhou, K. Zhang, and Y. Yu. 2013. Epidemiological investigation of community-acquired *Staphylococcus aureus* infection. Genet.Mol.Res. 12:6923-30.
48. Jones, R. N., J. E. Ross, M. Castanheira, and R. E. Mendes. 2008. United States resistance surveillance results for linezolid (LEADER Program for 2007). Diagn.Microbiol.Infect.Dis. 62:416-26.
49. Khanna, T., R. Friendship, C. Dewey, and J. S. Weese. 2008. Methicillin resistant *Staphylococcus aureus* colonization in pigs and pig farmers. Vet.Microbiol. 128:298-303.
50. Konvalinka, A., L. Errett, and I. W. Fong. 2006. Impact of treating *Staphylococcus aureus* nasal carriers on wound infections in cardiac surgery. J.Hosp.Infect. 64:162-8.
51. Koser, C. U., M. T. Holden, M. J. Ellington, E. J. Cartwright, N. M. Brown, A. L. Ogilvy-Stuart, L. Y. Hsu, C. Chewapreecha, N. J. Croucher, S. R. Harris, M. Sanders, M. C. Enright, G. Dougan, S. D. Bentley, J. Parkhill, L. J. Fraser, J. R. Betley, O. B. Schulz-Trieglaff, G. P. Smith, and S. J. Peacock. 2012. Rapid whole-genome sequencing for investigation of a neonatal MRSA outbreak. N.Engl.J.Med. 366:2267-75.
52. Kuroda, M., T. Ohta, I. Uchiyama, T. Baba, H. Yuzawa, I. Kobayashi, L. Cui, A. Oguchi, K. Aoki, Y. Nagai, J. Lian, T. Ito, M. Kanamori, H. Matsumaru, A. Maruyama, H. Murakami, A. Hosoyama, Y. Mizutani-Ui, N. K. Takahashi, T. Sawano, R. Inoue, C. Kaito, K. Sekimizu, H. Hirakawa, S. Kuhara, S. Goto, J. Yabuzaki, M. Kanehisa, A. Yamashita, K. Oshima, K. Furuya, C. Yoshino, T. Shiba, M. Hattori, N. Ogasawara, H. Hayashi, and K. Hiramatsu. 2001. Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*. Lancet 357:1225-40.
53. Kwan, T., J. Liu, M. DuBow, P. Gros, and J. Pelletier. 2005. The complete genomes and proteomes of 27 *Staphylococcus aureus* bacteriophages. Proc.Natl.Acad.Sci.U.S.A 102:5174-9.
54. Larsen, A. R., M. Stegger, S. Bocher, M. Sorum, D. L. Monnet, and R. L. Skov. 2009. Emergence and characterization of community-associated methicillin-resistant *Staphyloccocus aureus* infections in Denmark, 1999 to 2006. J.Clin.Microbiol. 47:73-8.
55. Li, M., X. Du, A. E. Villaruz, B. A. Diep, D. Wang, Y. Song, Y. Tian, J. Hu, F. Yu, Y. Lu, and M. Otto. 2012. MRSA epidemic linked to a quickly spreading colonization and virulence determinant. Nat.Med. 18:816-9.
56. Lim, K. T., C. S. Teh, M. Y. Yusof, and K. L. Thong. 2014. Mutations in *rpoB* and *fusA* cause resistance to rifampicin and fusidic acid in methicillin-resistant

Staphylococcus aureus strains from a tertiary hospital in Malaysia. Trans.R.Soc.Trop.Med.Hyg. 108:112-8.
57. Lim, S. K., H. M. Nam, G. C. Jang, H. S. Lee, S. C. Jung, and H. S. Kwak. 2012. The first detection of methicillin-resistant *Staphylococcus aureus* ST398 in pigs in Korea. Vet.Microbiol. 155:88-92.
58. Lindsay, J. A. 2010. Genomic variation and evolution of *Staphylococcus aureus*. Int.J.Med.Microbiol. 300:98-103.
59. Lindsay, J. A. 2014. *Staphylococcus aureus* genomics and the impact of horizontal gene transfer. Int.J.Med.Microbiol. 304:103-9.
60. Lindsay, J. A. and M. T. Holden. 2004. *Staphylococcus aureus*: superbug, super genome? Trends Microbiol. 12:378-85.
61. Lindsay, J. A., C. E. Moore, N. P. Day, S. J. Peacock, A. A. Witney, R. A. Stabler, S. E. Husain, P. D. Butcher, and J. Hinds. 2006. Microarrays reveal that each of the ten dominant lineages of *Staphylococcus aureus* has a unique combination of surface-associated and regulatory genes. J.Bacteriol. 188:669-76.
62. Liu, C., C. J. Graber, M. Karr, B. A. Diep, L. Basuino, B. S. Schwartz, M. C. Enright, S. J. O'Hanlon, J. C. Thomas, F. Perdreau-Remington, S. Gordon, H. Gunthorpe, R. Jacobs, P. Jensen, G. Leoung, J. S. Rumack, and H. F. Chambers. 2008. A population-based study of the incidence and molecular epidemiology of methicillin-resistant *Staphylococcus aureus* disease in San Francisco, 2004-2005. Clin.Infect.Dis. 46:1637-46.
63. Liu, Y., H. Wang, N. Du, E. Shen, H. Chen, J. Niu, H. Ye, and M. Chen. 2009. Molecular evidence for spread of two major methicillin-resistant *Staphylococcus aureus* clones with a unique geographic distribution in Chinese hospitals. Antimicrob.Agents Chemother. 53:512-8.
64. Lowy, F. D. 1998. *Staphylococcus aureus* infections. N.Engl.J.Med. 339:520-32.
65. Ma, X. X., D. D. Sun, S. Wang, M. L. Wang, M. Li, H. Shang, E. H. Wang, and E. J. Luo. 2011. Nasal carriage of methicillin-resistant *Staphylococcus aureus* among preclinical medical students: epidemiologic and molecular characteristics of methicillin-resistant *S. aureus* clones. Diagn.Microbiol.Infect.Dis. 70:22-30.
66. Maiques, E., C. Ubeda, M. A. Tormo, M. D. Ferrer, I. Lasa, R. P. Novick, and J. R. Penades. 2007. Role of staphylococcal phage and SaPI integrase in intra- and interspecies SaPI transfer. J.Bacteriol. 189:5608-16.
67. McCarthy, A. J., A. A. Witney, and J. A. Lindsay. 2012. *Staphylococcus aureus* temperate bacteriophage: carriage and horizontal gene transfer is lineage associated. Front Cell Infect.Microbiol. 2:6.
68. Melles, D. C., R. F. Gorkink, H. A. Boelens, S. V. Snijders, J. K. Peeters, M. J. Moorhouse, P. J. van der Spek, W. B. van Leeuwen, G. Simons, H. A. Verbrugh, and B. A. van. 2004. Natural population dynamics and expansion of pathogenic clones of *Staphylococcus aureus*. J.Clin.Invest 114:1732-40.
69. Moreno, F., C. Crisp, J. H. Jorgensen, and J. E. Patterson. 1995. Methicillin-resistant *Staphylococcus aureus* as a community organism. Clin.Infect.Dis. 21:1308-12.
70. Muileboom, J., M. Hamilton, K. Parent, D. Makahnouk, M. Kirlew, R. Saginur, F. Lam, and L. Kelly. 2013. Community-associated methicillin-resistant *Staphylococcus aureus* in northwest Ontario: A five-year report of incidence and antibiotic resistance. Can.J.Infect.Dis.Med.Microbiol. 24:e42-4.
71. Murray, N. E. 2000. Type I restriction systems: sophisticated molecular machines (a legacy of Bertani and Weigle). Microbiol.Mol.Biol.Rev. 64:412-34.
72. Neela, V., Z. A. Mohd, N. S. Mariana, B. A. van, Y. K. Liew, and E. G. Rad. 2009. Prevalence of ST9 methicillin-resistant *Staphylococcus aureus* among pigs and pig handlers in Malaysia. J.Clin.Microbiol. 47:4138-40.
73. Nichol, K. A., H. J. Adam, D. L. Roscoe, G. R. Golding, P. R. Lagace-Wiens, D. J.

Hoban, and G. G. Zhanel. 2013. Changing epidemiology of methicillin-resistant *Staphylococcus aureus* in Canada. J.Antimicrob.Chemother. 68 Suppl 1:i47-55.
74. Nulens, E., E. E. Stobberingh, D. H. van, S. Sebastian, F. H. van Tiel, P. S. Beisser, and R. H. Deurenberg. 2008. Molecular characterization of *Staphylococcus aureus* bloodstream isolates collected in a Dutch University Hospital between 1999 and 2006. J.Clin.Microbiol. 46:2438-41.
75. Ogston, A. 1880. Uber Abscesse. Arch Klin Chir 25:588-600.
76. Otto, M. 2008. Staphylococcal biofilms. Curr.Top.Microbiol.Immunol. 322:207-28.
77. Otto, M. 2014. *Staphylococcus aureus* toxins. Curr.Opin.Microbiol. 17:32-7.
78. Pinho, M. G., S. R. Filipe, L. H. de, and A. Tomasz. 2001. Complementation of the essential peptidoglycan transpeptidase function of penicillin-binding protein 2 (PBP2) by the drug resistance protein PBP2A in *Staphylococcus aureus*. J.Bacteriol. 183:6525-31.
79. Pourcel, C., K. Hormigos, L. Onteniente, O. Sakwinska, R. H. Deurenberg, and G. Vergnaud. 2009. Improved multiple-locus variable-number tandem-repeat assay for *Staphylococcus aureus* genotyping, providing a highly informative technique together with strong phylogenetic value. J.Clin.Microbiol. 47:3121-8.
80. Pu, W., Y. Su, J. Li, C. Li, Z. Yang, H. Deng, and C. Ni. 2014. High incidence of oxacillin-susceptible *mecA*-positive *Staphylococcus aureus* (OS-MRSA) associated with bovine mastitis in China. PLoS One 9:e88134.
81. Qiao, Y., F. Dong, W. Song, L. Wang, Y. Yang, and X. Shen. 2013. Hospital- and community-associated methicillin-resistant *Staphylococcus aureus*: a 6-year surveillance study of invasive infections in Chinese children. Acta Paediatr. 102:1081-6.
82. Qu, F., E. Cui, T. Guo, H. Li, S. Chen, L. Liu, W. Han, C. Bao, Y. Mao, and Y. W. Tang. 2010. Nasal colonization of and clonal transmission of methicillin-susceptible *Staphylococcus aureus* among Chinese military volunteers. J.Clin.Microbiol. 48:64-9.
83. Riley, T. V., J. W. Pearman, and I. L. Rouse. 1995. Changing epidemiology of methicillin-resistant *Staphylococcus aureus* in Western Australia. Med.J.Aust. 163:412-4.
84. Roberts, G. A., P. J. Houston, J. H. White, K. Chen, A. S. Stephanou, L. P. Cooper, D. T. Dryden, and J. A. Lindsay. 2013. Impact of target site distribution for Type I restriction enzymes on the evolution of methicillin-resistant *Staphylococcus aureus* (MRSA) populations. Nucleic Acids Res. 41:7472-84.
85. Robinson, D. A. and M. C. Enright. 2004. Evolution of *Staphylococcus aureus* by large chromosomal replacements. J.Bacteriol. 186:1060-4.
86. Rodriguez-Noriega, E., C. Seas, M. Guzman-Blanco, C. Mejia, C. Alvarez, L. Bavestrello, J. Zurita, J. Labarca, C. M. Luna, M. J. Salles, and E. Gotuzzo. 2010. Evolution of methicillin-resistant *Staphylococcus aureus* clones in Latin America. Int.J.Infect.Dis. 14:e560-6.
87. Schaumburg, F., A. S. Alabi, G. Peters, and K. Becker. 2014. New Epidemiology of *Staphylococcus aureus* infection in Africa. Clin.Microbiol.Infect. 20:589-96.
88. Schouls, L. M., E. C. Spalburg, L. M. van, X. W. Huijsdens, G. N. Pluister, M. G. van Santen-Verheuvel, H. G. van der Heide, H. Grundmann, M. E. Heck, and A. J. de Neeling. 2009. Multiple-locus variable number tandem repeat analysis of *Staphylococcus aureus*: comparison with pulsed-field gel electrophoresis and *spa*-typing. PLoS One 4:e5082.
89. Seeber, S., C. Kessler, and F. Gotz. 1990. Cloning, expression and characterization of the Sau3AI restriction and modification genes in *Staphylococcus carnosus* TM300. Gene 94:37-43.
90. Shambat, S., S. Nadig, S. Prabhakara, M. Bes, J. Etienne, and G. Arakere. 2012. Clonal complexes and virulence factors of *Staphylococcus aureus* from several

cities in India. BMC.Microbiol. 12:64.
91. Shore, A. C. and D. C. Coleman. 2013. Staphylococcal cassette chromosome *mec*: recent advances and new insights. Int.J.Med.Microbiol. 303:350-9.
92. Skov, R., K. Christiansen, S. J. Dancer, R. S. Daum, M. Dryden, Y. C. Huang, and F. D. Lowy. 2012. Update on the prevention and control of community-acquired meticillin-resistant *Staphylococcus aureus* (CA-MRSA). Int.J.Antimicrob.Agents 39:193-200.
93. Smith, T. C., M. J. Male, A. L. Harper, J. S. Kroeger, G. P. Tinkler, E. D. Moritz, A. W. Capuano, L. A. Herwaldt, and D. J. Diekema. 2009. Methicillin-resistant *Staphylococcus aureus* (MRSA) strain ST398 is present in midwestern U.S. swine and swine workers. PLoS One 4:e4258.
94. Song, J. H., P. R. Hsueh, D. R. Chung, K. S. Ko, C. I. Kang, K. R. Peck, J. S. Yeom, S. W. Kim, H. H. Chang, Y. S. Kim, S. I. Jung, J. S. Son, T. M. So, M. K. Lalitha, Y. Yang, S. G. Huang, H. Wang, Q. Lu, C. C. Carlos, J. A. Perera, C. H. Chiu, J. W. Liu, A. Chongthaleong, V. Thamlikitkul, and P. H. Van. 2011. Spread of methicillin-resistant *Staphylococcus aureus* between the community and the hospitals in Asian countries: an ANSORP study. J.Antimicrob.Chemother. 66:1061-9.
95. Song, Y., X. Du, T. Li, Y. Zhu, and M. Li. 2013. Phenotypic and molecular characterization of *Staphylococcus aureus* recovered from different clinical specimens of inpatients at a teaching hospital in Shanghai between 2005 and 2010. J.Med.Microbiol. 62:274-82.
96. Spoor, L. E., P. R. McAdam, L. A. Weinert, A. Rambaut, H. Hasman, F. M. Aarestrup, A. M. Kearns, A. R. Larsen, R. L. Skov, and J. R. Fitzgerald. 2013. Livestock origin for a human pandemic clone of community-associated methicillin-resistant *Staphylococcus aureus*. MBio. 4.
97. Strommenger, B., C. Braulke, D. Heuck, C. Schmidt, B. Pasemann, U. Nubel, and W. Witte. 2008. *spa* Typing of *Staphylococcus aureus* as a frontline tool in epidemiological typing. J.Clin.Microbiol. 46:574-81.
98. Teo, J., T. Y. Tan, P. Y. Hon, W. Lee, T. H. Koh, P. Krishnan, and L. Y. Hsu. 2013. ST22 and ST239 MRSA duopoly in Singaporean hospitals: 2006-2010. Epidemiol.Infect. 141:153-7.
99. Thompson, D. S., R. Workman, and M. Strutt. 2009. Decline in the rates of meticillin-resistant *Staphylococcus aureus* acquisition and bacteraemia in a general intensive care unit between 1996 and 2008. J.Hosp.Infect. 71:314-9.
100. Udo, E. E., J. W. Pearman, and W. B. Grubb. 1993. Genetic analysis of community isolates of methicillin-resistant *Staphylococcus aureus* in Western Australia. J.Hosp.Infect. 25:97-108.
101. Van den Eede, A., A. Martens, U. Lipinska, M. Struelens, A. Deplano, O. Denis, F. Haesebrouck, F. Gasthuys, and K. Hermans. 2009. High occurrence of methicillin-resistant *Staphylococcus aureus* ST398 in equine nasal samples. Vet.Microbiol. 133:138-44.
102. van, B. A., D. C. Melles, J. K. Peeters, W. B. van Leeuwen, D. E. van, X. W. Huijsdens, E. Spalburg, A. J. de Neeling, and H. A. Verbrugh. 2008. Methicillin-resistant and -susceptible *Staphylococcus aureus* sequence type 398 in pigs and humans. Emerg.Infect.Dis. 14:479-83.
103. van, B. A., H. G. Niesters, W. G. MacKay, and W. B. van Leeuwen. 2007. Quality control of direct molecular diagnostics for methicillin-resistant *Staphylococcus aureus*. J.Clin.Microbiol. 45:2698-700.
104. van, B. A., N. J. Verkaik, C. P. de Vogel, H. A. Boelens, J. Verveer, J. L. Nouwen, H. A. Verbrugh, and H. F. Wertheim. 2009. Reclassification of *Staphylococcus aureus* nasal carriage types. J.Infect.Dis. 199:1820-6.
105. van, L., I, X. Huijsdens, E. Tiemersma, N. A. de, N. Sande-Bruinsma, D. Beaujean,

A. Voss, and J. Kluytmans. 2007. Emergence of methicillin-resistant *Staphylococcus aureus* of animal origin in humans. Emerg.Infect.Dis. 13:1834-9.

106. von, E. C., K. Becker, K. Machka, H. Stammer, and G. Peters. 2001. Nasal carriage as a source of *Staphylococcus aureus* bacteremia. Study Group. N.Engl.J.Med. 344:11-6.
107. Vos, M. C., M. D. Behrendt, D. C. Melles, F. P. Mollema, G. W. de, G. Parlevliet, A. Ott, D. Horst-Kreft, B. A. van, and H. A. Verbrugh. 2009. 5 years of experience implementing a methicillin-resistant *Staphylococcus aureus* search and destroy policy at the largest university medical center in the Netherlands. Infect.Control Hosp.Epidemiol. 30:977-84.
108. Wagenaar, J. A., H. Yue, J. Pritchard, M. Broekhuizen-Stins, X. Huijsdens, D. J. Mevius, T. Bosch, and D. E. Van. 2009. Unexpected sequence types in livestock associated methicillin-resistant *Staphylococcus aureus* (MRSA): MRSA ST9 and a single locus variant of ST9 in pig farming in China. Vet.Microbiol. 139:405-9.
109. Waldron, D. E. and J. A. Lindsay. 2006. Sau1: a novel lineage-specific type I restriction-modification system that blocks horizontal gene transfer into *Staphylococcus aureus* and between *S. aureus* isolates of different lineages. J.Bacteriol. 188:5578-85.
110. Wang, L., Y. Liu, Y. Yang, G. Huang, C. Wang, L. Deng, Y. Zheng, Z. Fu, C. Li, Y. Shang, C. Zhao, M. Sun, X. Li, S. Yu, K. Yao, and X. Shen. 2012. Multidrug-resistant clones of community-associated meticillin-resistant *Staphylococcus aureus* isolated from Chinese children and the resistance genes to clindamycin and mupirocin. J.Med.Microbiol. 61:1240-7.
111. Wang, R., K. R. Braughton, D. Kretschmer, T. H. Bach, S. Y. Queck, M. Li, A. D. Kennedy, D. W. Dorward, S. J. Klebanoff, A. Peschel, F. R. DeLeo, and M. Otto. 2007. Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. Nat.Med. 13:1510-4.
112. Wang, X., J. Meng, T. Zhou, Y. Zhang, B. Yang, M. Xi, J. Sheng, S. Zhi, and X. Xia. 2012. Antimicrobial susceptibility testing and genotypic characterization of *Staphylococcus aureus* from food and food animals. Foodborne.Pathog.Dis. 9:95-101.
113. Wertheim, H. F., D. C. Melles, M. C. Vos, L. W. van, B. A. van, H. A. Verbrugh, and J. L. Nouwen. 2005. The role of nasal carriage in *Staphylococcus aureus* infections. Lancet Infect.Dis. 5:751-62.
114. Wertheim, H. F., M. C. Vos, A. Ott, B. A. van, A. Voss, J. A. Kluytmans, P. H. van Keulen, C. M. Vandenbroucke-Grauls, M. H. Meester, and H. A. Verbrugh. 2004. Risk and outcome of nosocomial *Staphylococcus aureus* bacteraemia in nasal carriers versus non-carriers. Lancet 364:703-5.
115. Williamson, D. A., G. W. Coombs, and G. R. Nimmo. 2014. *Staphylococcus aureus* "Down Under': Contemporary epidemiology of *Staphylococcus aureus* in Australia, New Zealand and the South West Pacific. Clin.Microbiol.Infect. 20:597-604.
116. Wu, D., Q. Wang, Y. Yang, W. Geng, Q. Wang, S. Yu, K. Yao, L. Yuan, and X. Shen. 2010. Epidemiology and molecular characteristics of community-associated methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* from skin/soft tissue infections in a children's hospital in Beijing, China. Diagn.Microbiol.Infect.Dis. 67:1-8.
117. Xiao Yonghong, Shen Ping, Wei Zeqing, Chen Yunbo, Kong Haishen, Yang Qing, Zhang Weili, Chen Xiao, and Li Lanjuan. 2011. Mohnarin report of 2010: surveillance of bacterial resistance in China. Chin J Nosocomio. 21: 4896-902 (Chinese).
118. Xiao Yonghong, Shen Ping, Wei Zeqing, Chen Yunbo, Kong Haishen, Yang Qing, Zhang Weili, Chen Xiao, and Li Lanjuan. 2012. Mohnarin report of 2011:

surveillance of bacterial resistance in China. Chin J Nosocomio. 22:4946-52 (Chinese).
119. Xiao, M., H. Wang, Y. Zhao, L. L. Mao, M. Brown, Y. S. Yu, M. V. O'Sullivan, F. Kong, and Y. C. Xu. 2013. National surveillance of methicillin-resistant *Staphylococcus aureus* in China highlights a still-evolving epidemiology with 15 novel emerging multilocus sequence types. J.Clin.Microbiol. 51:3638-44.
120. Xiao, Y. H., C. G. Giske, Z. Q. Wei, P. Shen, A. Heddini, and L. J. Li. 2011. Epidemiology and characteristics of antimicrobial resistance in China. Drug Resist.Updat. 14:236-50.
121. Xu, B. L., G. Zhang, H. F. Ye, E. J. Feil, G. R. Chen, X. M. Zhou, X. M. Zhan, S. M. Chen, and W. B. Pan. 2009. Predominance of the Hungarian clone (ST 239-III) among hospital-acquired meticillin-resistant *Staphylococcus aureus* isolates recovered throughout mainland China. J.Hosp.Infect. 71:245-55.
122. Xu, S. Y., A. R. Corvaglia, S. H. Chan, Y. Zheng, and P. Linder. 2011. A type IV modification-dependent restriction enzyme SauUSI from *Staphylococcus aureus* subsp. aureus USA300. Nucleic Acids Res. 39:5597-610.
123. Yao, D., F. Y. Yu, Z. Q. Qin, C. Chen, S. S. He, Z. Q. Chen, X. Q. Zhang, and L. X. Wang. 2010. Molecular characterization of *Staphylococcus aureus* isolates causing skin and soft tissue infections (SSTIs). BMC.Infect.Dis. 10:133.
124. Yu, F., Z. Chen, C. Liu, X. Zhang, X. Lin, S. Chi, T. Zhou, Z. Chen, and X. Chen. 2008. Prevalence of *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes among isolates from hospitalised patients in China. Clin.Microbiol.Infect. 14:381-4.
125. Yu, V. L., A. Goetz, M. Wagener, P. B. Smith, J. D. Rihs, J. Hanchett, and J. J. Zuravleff. 1986. *Staphylococcus aureus* nasal carriage and infection in patients on hemodialysis. Efficacy of antibiotic prophylaxis. N.Engl.J.Med. 315:91-6.
126. Zhanel, G. G., M. DeCorby, H. Adam, M. R. Mulvey, M. McCracken, P. Lagace-Wiens, K. A. Nichol, A. Wierzbowski, P. J. Baudry, F. Tailor, J. A. Karlowsky, A. Walkty, F. Schweizer, J. Johnson, and D. J. Hoban. 2010. Prevalence of antimicrobial-resistant pathogens in Canadian hospitals: results of the Canadian Ward Surveillance Study (CANWARD 2008). Antimicrob.Agents Chemother. 54:4684-93.
127. Zhang, C., L. Song, H. Chen, Y. Liu, Y. Qin, and Y. Ning. 2012. Antimicrobial susceptibility and molecular subtypes of *Staphylococcus aureus* isolated from pig tonsils and cow's milk in China. Can.J.Vet.Res. 76:268-74.
128. Zhang, W., Z. Hao, Y. Wang, X. Cao, C. M. Logue, B. Wang, J. Yang, J. Shen, and C. Wu. 2011. Molecular characterization of methicillin-resistant *Staphylococcus aureus* strains from pet animals and veterinary staff in China. Vet.J. 190:e125-9.
129. Zhao, C., Y. Liu, M. Zhao, Y. Liu, Y. Yu, H. Chen, Q. Sun, H. Chen, W. Jiang, Y. Liu, S. Han, Y. Xu, M. Chen, B. Cao, and H. Wang. 2012. Characterization of community acquired *Staphylococcus aureus* associated with skin and soft tissue infection in Beijing: high prevalence of PVL+ ST398. PLoS One 7:e38577.

Chapter 2

Increasing Resistance in Multiresistant Methicillin-Resistant *Staphylococcus aureus* Clones Isolated from a Chinese Hospital over a Five-Year Period

Xiaomei Yan, Xiaoxia Tao, Lihua He, Zhigang Cui, Jianzhong Zhang

Published in *Microbial Drug Resistance* (2011) 17, 235-9

Abstract

The aim was to study the changes in the antimicrobial resistance of MRSA clones over a five-year period (from 2000 to 2005) at a representative hospital in Beijing, China. A total of 100 randomly selected MRSA strains were analyzed using antimicrobial susceptibility testing, pulsed-field gel electrophoresis (PFGE), spa typing, MLST, SCC*mec* typing and PCR for the Panton-Valentine leukocidin (PVL) virulence factor. Resistance to rifampin increased greatly from 32% (16/50) to 68% (34/50). High-level mupirocin-resistant isolates were found only in 2005, when four were identified. Intermediate susceptibly to quinupristin-dalfopristin increased from 22% (11/50) to 52% (26/50) between 2000 and 2005. The main antimicrobial resistance profiles changed from TC-GM-CI-EM-CM in 2000 to TC-GM-CI-EM-CM-RI in 2005. The main PFGE type changed from Types C, L and E in 2000 to types J, F and N in 2005. ST239-MRSA-III was the most predominant clone in 2000 and 2005, while ST5-MRSA-II was found only in 2005. There were increasing levels of antimicrobial resistance and epidemiological changes in the HA-MRSA strains isolated in this facility between 2000 and 2005.

INTRODUCTION

Staphylococcus aureus（*S .aureus*）has became as a very important pathogen that causes several diseases, ranging from minor infections of the skin to wound infections, bacteremia and necrotizing pneumonia. The epidemiological characteristics of *S. aureus*, especially methicillin-resistant *S. aureus* (MRSA), are changing rapidly. From the fist MRSA strain reported until the present, various hospital-associated MRSA (HA-MRSA) clones have been disseminated worldwide (3, 5, 6, 12, 26). The emergence of new strains of community-associated MRSA (CA-MRSA) in many parts of the world is well documented (15, 21) and causes an increasing proportion of healthcare-associated infections (13).

In China, MRSA is highly endemic in hospitals with mean prevalence rates of >50% in 2005(33).Two major epidemic MRSA clones with a unique geographic distribution across China, ST239-MRSA-III and ST5-MRSA- II have been reported recently (22). Multilocus sequence typing (MLST) for a few isolates over five years was completed by us and provided an important clue (34). In order to investigate the dynamics of MRSA over long time periods, we characterized MRSA isolates from a Chinese hospital collected between 2000 and 2005 to identify changes in antimicrobial resistance, molecular typing and virulence factors.

MATERIALS AND METHODS

Isolates

A total of 100 MRSA strains were isolated from several clinical sources (including the respiratory tract (n=76), blood (n=3), drainage (n=8), pus (=7), wounds (n=3), and other sources (n =3) that were randomly selected from Beijing Union Medical Hospital, which has about 1800 beds. Fifty strains were isolated in 2000, and 50 more were isolated in 2005. All strains were confirmed by PCR analysis of the *mecA* and *nuc* genes (4).

Antimicrobial susceptibility tests

A total of 14 antimicrobial agents were tested, including linezolid, chloramphenicol, tetracycline, gentamicin, vancomycin, ciprofloxacin, erythromycin, clindamycin, rifampin, quinupristin-dalfopristin, teicoplanin, mupirocin, fusidic acid and trimethoprim. Vancomycin was tested by broth dilution according to CLSI, and the other antimicrobial agents were tested by Etest (AB Biodisk, Solna, Sweden). CLSI breakpoints were used for MIC interpretation. The resistance breakpoints for mupirocin and fusidic acid were defined as described previously (9, 23).

Molecular typing

All isolates were investigated by pulsed-field gel electrophoresis (PFGE), SCC*mec* typing and *spa* typing. Multilocus sequence typing was performed on twenty-nine strains that included representatives of each spa type. Genomic DNA and plasmids were extracted using the DNeasy blood and tissue kit and the QIAprep Spin Miniprep kit respectively (Qiagen GmbH, Hilden, German). Amplifications were performed in a Mycycler^{TM} thermal cycler (Bio-Rad, United States). PFGE was performed using the CHEF-DR III System (Bio-Rad, United States).

PFGE using *Sma*I was set up according to the America CDC Standardized Protocol for Molecular Subtyping of *Listeria monocytogenes* by PFGE (http:// www.cdc.gov/PULSENET/protocols.htm).The MRSA suspension incorporated into the agarose block was standardized to an optical density (OD) of 5.5–6.5, and 2 µl lysostaphin (1 mg/mL) was added to 250 µl of bacterial suspension, which was immediately mixed with an equal volume of 1% low-melting-point agarose. Digital images were analyzed by BioNumerics software (v. 4.6, Bio-Rad) using the Dice coefficient and were represented by unweighted pair group method arithmetic averages (UPGMA) with 1.5% tolerance and 1% optimization settings. A similarity cutoff of 80% and the criterion of a difference of 6 bands as described by Tenover (30) were used to define a cluster. Isolates showing identical or related PFGE patterns were considered to belong to the same clone. Clones were labeled with a capital letter (A, B, C, and so on), and related profiles were indicated by adding a number (A1, A2, B1, B2, and so on).

Spa typing, MLST and SCC*mec* typing were performed as previously described(7,8,14,27,35).

***pvl and mupA* genes detection** Panton–Valentine leukocidin (PVL) genes (lukS-PV and lukF-PV) were detected by PCR(21). The high-level mupirocin-resistant isolates (based on the Etest method) were detected by PCR for the *mupA* gene as described previously (1).

Statistical analysis

Statistical analysis was performed with the SPSS software package using chi-square and Fisher's exact tests.

RESULTS

Antimicrobial susceptibility testing

Isolates from the various time periods did not demonstrate resistance or increases in the MIC of vancomycin, teicoplanin and linezolid. Resistance to rifampin increased greatly from 32%

(16/50) to 68% (34/50, p<0.001). Four high-level mupirocin-resistant isolates were found only in samples from 2005. Intermediate susceptibility rates to quinupristin-dalfopristin increased from 22% (11/50) to 52% (26/50, p<0.01) from 2000 to 2005. Resistance to ciprofloxacin was universal. Resistance to chloramphenicol, tetracycline, erythromycin and clindamycin increased slightly from 4% to 6%, 94% to 98%, 98% to 100% and 98% to 100%, respectively. On the other hand, gentamicin, quinupristin-dalfopristin, fusidic acid and trimethoprim resistance decreased from 100% to 94%, 12% to 8%, 4% to 0% and 4% to 0% respectively.

Antibiotic resistance profiles and molecular epidemiology

Overall, fifteen PFGE strain types (A through O) and four clusters (1 through 4) were identified among the 100 MRSA isolates. Five PFGE types, type (C, J, N, F, and L) were the dominant types, constituting 82% of all isolates. Types C, L and E were the dominant types from isolates collected in 2000, accounting for 88% (44/50), while types J, F and N were the dominant types in 2005, accounting for 74% (37/50).

Eleven antimicrobial resistant profiles were identified in total. Two predominant profiles (TC-GM-CI-EM-CM-RI and TC-GM-CI-EM-CM) contained 47 and 40 isolates, respectively. The antimicrobial resistance profile TC-GM-CI-EM-CM-RI included 14 isolates in 2000, corresponding to PFGE types E, L and M, and included 33 isolates in 2005, corresponding to PFGE types A, F, I, J, K, L and N. The antimicrobial resistance profile TC-GM-CI-EM-CM included 29 isolates in 2000, corresponding to PFGE types C,D and N, and included 11 isolates in 2005, corresponding to PFGE types C, F, G and L (Table 1).

Typing all isolates yielded five *spa* types (Table 1). T037 was the predominant *spa* type in 2000, accounting for 74% (37/50) and the second most prominent was t030, accounting for 24% (12/50). While t030 was the most predominant *spa* type in 2005, accounting for 66% (33/50); the second was t002, accounting for 30% (15/50, p < 0.001).

Twenty-nine strains that included representatives of each *spa* type yielded three STs belonging to two clonal complexes (Table 1). ST239 was the predominant ST, which belonged to clonal complex CC8 and constitutes 72.41% of isolates (21/29). The second ST was ST5, which belonged to clonal complex CC5 and constitutes 24.14% of isolates (7/29). ST5 was only found in 2005.

From all isolates, two SCC*mec* types were identified, namely II and III (Table 1). The most common SCC*mec* type was type III, which was found in 80 isolates (80/100 or 80%), including 45 isolates from 2000 and 35 isolates (35/50 or 70%) from 2005. The second was type II, which was only found in 15 isolates (15/100 or 15%) from 2005. A total of five isolates

(5/100 or 5%) were nontypeable by the multiplex SCC*mec* typing method.

Table 1. MLST, *spa*, SCC*mec* and PFGE types of MRSA isolates and their antibiotic resistance profiles in 2000 and 2005

ST(CC)	*spa type*	*SCCmec*	*PFGE type*	*antibiotic resistance profile*[a]	*% of isolates in 2000*	*% of isolates in 2005*
5(5)	t002	II	F	TC-GM-CI-EM-CM	0	16
				CL-TC-GM-CI-EM-CM-MU	0	4
			G	TC-GM-CI-EM-CM	0	2
			H	TC-CI-EM-CM-MU	0	2
				CL-TC-GM-CI-EM-CM-MU	0	2
				TC-CI-EM-CM	0	2
			L1	TC-GM-CI-EM-CM	0	2
72(8)	t148	unknown	O	GM-CI-EM-CM	2	0
239(8)	t1152	III	A	TC-GM-CI-EM-CM-RI	0	2
	t037	III	B	CL-TC-GM-CI-EM-CM-RI-QDA-MU-FU-TR	2	0
			C1	TC-GM-CI-EM-CM	50	2
				TC-GM-CI-EM-CM-FU-TR	2	0
				GM-CI-EM-CM	2	0
			C2	TC-GM-CI-EM-CM	4	0
				GM-CI-EM-CM	2	0
			D	TC-GM-CI-EM-CM	2	0

		E	CL-TC-GM-CI-EM-CM	2	0
	unknown	E	TC-GM-CI-EM-CM-RI	8	0
t030	III	F	TC-GM-CI-EM-CM-RI	0	2
		I1	TC-GM-CI-EM-CM-RI	0	6
		I2	TC-GM-CI-EM-CM-RI	0	2
		J1	TC-GM-CI-EM-CM-RI	0	6
		J2	TC-GM-CI-EM-CM-RI	0	22
			TC-CI-EM-CM-RI	0	2
		K	TC-GM-CI-EM-CM-RI	0	2
		L1	TC-GM-CI-EM-CM-RI-QDA	2	0
			TC-GM-CI-EM-CM-RI	16	0
		L2	TC-GM-CI-EM-CM-RI	0	2
		M	TC-GM-CI-EM-CM-RI	4	0
		N1	TC-GM-CI-EM-CM-RI	0	4
		N2	TC-GM-CI-EM-CM	2	0
			TC-GM-CI-EM-CM-RI	0	18

[a]TC, Tetracycline ;CL, Chloramphenicol;GM, Gentamicin;CI, Ciprofloxacin;EM, Erythromycin;CM, Clindamycin;RI, Rifampin; QDA,Quinupristin/Dalfopristin; MU, Mupirocin;FU, Fusidic acid;TR, Trimethoprim

Detection of *pvl* and *mupA*

No isolate was *pvl* positive. The four high-level mupirocin-resistant isolates were all positive for *mupA*.

Discussion

MRSA strains associated with community acquisition were not found to have been introduced

into this hospital, but there were epidemiological changes in the types of HA-MRSA strains in the facility over the five years included in our study, with an associated change in antimicrobial resistance patterns.

The main antimicrobial resistance profile changed from TC-GM-CI-EM-CM in 2000 to TC-GM-CI-EM-CM-RI in 2005. Resistance to rifampin increased from 32% to 68%, and the MIC_{50} for rifampin increased from 0.008 to 256μg/ml. Combination therapy with rifampin has been used to treat *S aureus* infections (24,29) and has been widely used in China in recent years to treat esophagitis, dysentery (difficult to cure or resistant), skin infections, hordeolum, purulent otitis media and mycoplasma pneumonia. From our data, we found that an increasing number of resistant isolates are emerging with the wide spread use of rifampin.

The data in this study revealed that resistance to mupirocin increased from 2% to 8%. We found four high-level-resistant (MupRH) isolates with *mupA* in 2005 (11), which were PFGE types F and H, belonged to the same spa type and STs (t002, ST5-MRSA-II), and belonged to two antimicrobial resistant profiles (CL-TC-GM-CI-EM-CM-MU and TC-CI-EM-CM-MU). One low-level-resistant (MupRL)(2,10) isolate from 2000 was PFGE type B, t037, ST239-MRSA-III, and resistant to 11 antimicrobial drugs (CL-TC-GM-CI-EM-CM-RI-QDA-MU-FU-TR). This result indicated the MupRL isolates and the MupRH isolate were different clones. Beginning in December 2005, mupirocin could be purchased OTC in China. From the experience in other countries (31), more attention to it should be paid to this trend. Caution should be taken whenever high-level resistance strains are detected and control measures should be implemented because transmissible plasmids have led to outbreaks (20).

The other interesting finding was that 10% isolates were resistant to quinupristin-dalfopristin while 37% (37/100) of isolates displayed intermediate susceptibility to the drug.. The rate of intermediate susceptibility to quinupristin-dalfopristin increased from 22% (11/50) to 52% (26/50) between 2000 and 2005. We are quite curious how this has occurred because quinupristin-dalfopristin had not been marketed in China until the present. Virginiamycin, another streptogramin A/B combination, has long been used in animal feed as a growth promoter in many countries, including China. This type of use selects for virginiamycin-resistant strains of *E. faecium*, which are cross-resistant to quinupristin-dalfopristin (18) and which may pose a risk to public health. As far as we know, staphylococci are generally host-specific, although some exceptions have been noted, such as MRSA from pigs that can colonize humans, as observed in the Netherlands (16). Thus, whether these isolates resistant to quinupristin-dalfopristin originated from animals needs to

be investigated further.

ST5-MRSA-II was a new type found in the hospital in 2005; this strain has also spread widely in European countries and is the predominant MRSA clone in Korea and Japan (19). Some published data have shown that clonal evolution can occur within a single hospital. In a Mexico City hospital, a local clone (ST30-MRSA-IV) was predominant between 1997 and 2000, but it was completely replaced over a two-year period by the New York/Japan clone (ST5-MRSA-II) (32). Similarly, a study in Spain showed that ST247-MRSA-I was replaced by ST36-MRSA-II between 1998 and 2002 (28). The ST5-MRSA-II clone emerged after 2000 and was not found previously in Hong Kong (17). These results indicate that ST5-MRSA-II has become more common in this Beijing hospital in recently years. Recently, research on the ST5-MRSA clone has provided strong evidence that the geographical spread of MRSA over long distances and across cultural borders is a rare event compared with the frequency with which the staphylococcal cassette chromosome island has been imported (25). Thus, continuous surveillance of MRSA and methicillin-sensitive *staphylococcus aureus* (MSSA) in hospitals and communities is of great importance for understanding the local epidemiology of MRSA.

Conclusions

Our study showed that there were increasing levels of antimicrobial resistance and epidemiological changes in the HA-MRSA strains collected in the facility between 2000 and 2005.

Acknowledgement

This work was supported by "major infectious diseases such as AIDS and viral hepatitis prevention and control" technology major projects（ 2008ZX10004－002 ）from the Ministry of Science and Technology.

We thank Beijing Union Medical Hospital for the MRSA isolates supported.

Reference List

1. Anthony, R. M., A. M. Connor, E. G. Power, and G. L. French. 1999. Use of the polymerase chain reaction for rapid detection of high-level mupirocin resistance in staphylococci. Eur.J.Clin.Microbiol.Infect.Dis. 18:30-4.
2. Antonio, M., N. McFerran, and M. J. Pallen. 2002. Mutations affecting the Rossman fold of isoleucyl-tRNA synthetase are correlated with low-level mupirocin resistance in *Staphylococcus aureus*. Antimicrob.Agents Chemother. 46:438-42.
3. Aparicio, P., J. Richardson, S. Martin, A. Vindel, R. R. Marples, and B. D. Cookson. 1992. An epidemic methicillin-resistant strain of *Staphylococcus aureus* in Spain. Epidemiol.Infect. 108:287-98.
4. Brakstad, O. G., K. Aasbakk, and J. A. Maeland. 1992. Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the *nuc* gene. J.Clin.Microbiol. 30:1654-60.
5. de, L. H., E. P. Severina, H. Milch, M. K. Thege, and A. Tomasz. 1997. Wide geographic distribution of a unique methicillin-resistant *Staphylococcus aureus* clone in Hungarian hospitals. Clin.Microbiol.Infect. 3:289-96.
6. Deplano, A., W. Witte, W. J. van Leeuwen, Y. Brun, and M. J. Struelens. 2000. Clonal dissemination of epidemic methicillin-resistant *Staphylococcus aureus* in Belgium and neighboring countries. Clin.Microbiol.Infect. 6:239-45.
7. Enright, M. C., N. P. Day, C. E. Davies, S. J. Peacock, and B. G. Spratt. 2000. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. J.Clin.Microbiol. 38:1008-15.
8. Feil, E. J., B. C. Li, D. M. Aanensen, W. P. Hanage, and B. G. Spratt. 2004. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J.Bacteriol. 186:1518-30.
9. Finlay, J. E., L. A. Miller, and J. A. Poupard. 1997. Interpretive criteria for testing susceptibility of staphylococci to mupirocin. Antimicrob.Agents Chemother. 41:1137-9.
10. Fujimura, S., Y. Tokue, and A. Watanabe. 2003. Isoleucyl-tRNA synthetase mutations in *Staphylococcus aureus* clinical isolates and in vitro selection of low-level mupirocin-resistant strains. Antimicrob.Agents Chemother. 47:3373-4.
11. Gilbart, J., C. R. Perry, and B. Slocombe. 1993. High-level mupirocin resistance in *Staphylococcus aureus*: evidence for two distinct isoleucyl-tRNA synthetases. Antimicrob.Agents Chemother. 37:32-8.
12. Gomes, A. R., I. S. Sanches, S. M. ires de, E. Castaneda, and L. H. de. 2001. Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* in Colombian hospitals: dominance of a single unique multidrug-resistant clone. Microb.Drug Resist. 7:23-32.
13. Gonzalez, B. E., A. M. Rueda, S. A. Shelburne, III, D. M. Musher, R. J. Hamill, and K. G. Hulten. 2006. Community-associated strains of methicillin-resistant *Staphylococccus aureus* as the cause of healthcare-associated infection. Infect.Control Hosp.Epidemiol. 27:1051-6.
14. Harmsen, D., H. Claus, W. Witte, J. Rothganger, H. Claus, D. Turnwald, and U. Vogel. 2003. Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for *spa* repeat determination and database management. J.Clin.Microbiol. 41:5442-8.
15. Herold, B. C., L. C. Immergluck, M. C. Maranan, D. S. Lauderdale, R. E. Gaskin, S. Boyle-Vavra, C. D. Leitch, and R. S. Daum. 1998. Community-acquired methicillin-resistant *Staphylococcus aureus* in children with no identified predisposing risk. JAMA. 279:593-8.
16. Huijsdens, X. W., B. J. van Dijke, E. Spalburg, M. G. van Santen-Verheuvel, M. E. Heck, G. N. Pluister, A. Voss, W. J. Wannet, and A. J. de Neeling. 2006.

Community-acquired MRSA and pig-farming. Ann.Clin.Microbiol.Antimicrob. 5:26.
17. Ip, M., R. W. Yung, T. K. Ng, W. K. Luk, C. Tse, P. Hung, M. Enright, and D. J. Lyon. 2005. Contemporary methicillin-resistant *Staphylococcus aureus* clones in Hong Kong. J.Clin.Microbiol. 43:5069-73.
18. Jensen, L. B., A. M. Hammerum, F. M. Aerestrup, A. E. van den Bogaard, and E. E. Stobberingh. 1998. Occurrence of *satA* and *vgb* genes in streptogramin-resistant Enterococcus faecium isolates of animal and human origins in the Netherlands. Antimicrob.Agents Chemother. 42:3330-1.
19. Ko, K. S., J. Y. Lee, J. Y. Suh, W. S. Oh, K. R. Peck, N. Y. Lee, and J. H. Song. 2005. Distribution of major genotypes among methicillin-resistant *Staphylococcus aureus* clones in Asian countries. J.Clin.Microbiol. 43:421-6.
20. Leski, T. A., M. Gniadkowski, A. Skoczynska, E. Stefaniuk, K. Trzcinski, and W. Hryniewicz. 1999. Outbreak of mupirocin-resistant staphylococci in a hospital in Warsaw, Poland, due to plasmid transmission and clonal spread of several strains. J.Clin.Microbiol. 37:2781-8.
21. Lina, G., Y. Piemont, F. Godail-Gamot, M. Bes, M. O. Peter, V. Gauduchon, F. Vandenesch, and J. Etienne. 1999. Involvement of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. Clin.Infect.Dis. 29:1128-32.
22. Liu, Y., H. Wang, N. Du, E. Shen, H. Chen, J. Niu, H. Ye, and M. Chen. 2009. Molecular evidence for spread of two major methicillin-resistant *Staphylococcus aureus* clones with a unique geographic distribution in Chinese hospitals. Antimicrob.Agents Chemother. 53:512-8.
23. MacGowan, A. P. and R. Wise. 2001. Establishing MIC breakpoints and the interpretation of in vitro susceptibility tests. J.Antimicrob.Chemother. 48 Suppl 1:17-28.
24. Miller, L. G., C. Quan, A. Shay, K. Mostafaie, K. Bharadwa, N. Tan, K. Matayoshi, J. Cronin, J. Tan, G. Tagudar, and A. S. Bayer. 2007. A prospective investigation of outcomes after hospital discharge for endemic, community-acquired methicillin-resistant and -susceptible *Staphylococcus aureus* skin infection. Clin.Infect.Dis. 44:483-92.
25. Nubel, U., P. Roumagnac, M. Feldkamp, J. H. Song, K. S. Ko, Y. C. Huang, G. Coombs, M. Ip, H. Westh, R. Skov, M. J. Struelens, R. V. Goering, B. Strommenger, A. Weller, W. Witte, and M. Achtman. 2008. Frequent emergence and limited geographic dispersal of methicillin-resistant *Staphylococcus aureus*. Proc.Natl.Acad.Sci.U.S.A 105:14130-5.
26. Oliveira, D., I. Santos-Sanches, R. Mato, M. Tamayo, G. Ribeiro, D. Costa, and L. H. de. 1998. Virtually all methicillin-resistant *Staphylococcus aureus* (MRSA) infections in the largest Portuguese teaching hospital are caused by two internationally spread multiresistant strains: the 'Iberian' and the 'Brazilian' clones of MRSA. Clin.Microbiol.Infect. 4:373-84.
27. Oliveira, D. C. and L. H. de. 2002. Multiplex PCR strategy for rapid identification of structural types and variants of the *mec* element in methicillin-resistant *Staphylococcus aureus*. Antimicrob.Agents Chemother. 46:2155-61.
28. Perez-Roth, E., F. Lorenzo-Diaz, N. Batista, A. Moreno, and S. Mendez-Alvarez. 2004. Tracking methicillin-resistant *Staphylococcus aureus* clones during a 5-year period (1998 to 2002) in a Spanish hospital. J.Clin.Microbiol. 42:4649-56.
29. Schwalm, J. D., P. El-Helou, and C. H. Lee. 2004. Clinical outcome with oral linezolid and rifampin following recurrent methicillin-resistant *Staphylococcus aureus* bacteremia despite prolonged vancomycin treatment. Can.J.Infect.Dis. 15:97-100.
30. Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan. 1995. Interpreting chromosomal DNA restriction

patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J.Clin.Microbiol. 33:2233-9.

31. Upton, A., S. Lang, and H. Heffernan. 2003. Mupirocin and *Staphylococcus aureus*: a recent paradigm of emerging antibiotic resistance. J.Antimicrob.Chemother. 51:613-7.
32. Velazquez-Meza, M. E., S. M. ires de, G. Echaniz-Aviles, F. Solorzano-Santos, G. Miranda-Novales, J. Silva-Sanchez, and L. H. de. 2004. Surveillance of methicillin-resistant *Staphylococcus aureus* in a pediatric hospital in Mexico City during a 7-year period (1997 to 2003): clonal evolution and impact of infection control. J.Clin.Microbiol. 42:3877-80.
33. Wang, H., Y. Liu, H. Sun, Y. Xu, X. Xie, and M. Chen. 2008. In vitro activity of ceftobiprole, linezolid, tigecycline, and 23 other antimicrobial agents against *Staphylococcus aureus* isolates in China. Diagn.Microbiol.Infect.Dis. 62:226-9.
34. Yan Xiaomei, Gu Yixin, He Lihua, and Zhang Jianzhong. 2009. Multilocus Sequence Typing of Methicillin-resistant *Staphylococcus aureus*. Chinese journal of preventive medicine 2:137-40.
35. Zhang, K., J. A. McClure, S. Elsayed, T. Louie, and J. M. Conly. 2005. Novel multiplex PCR assay for characterization and concomitant subtyping of staphylococcal cassette chromosome *mec* types I to V in methicillin-resistant *Staphylococcus aureus*. J.Clin.Microbiol. 43:5026-33.

Chapter 3

Characterization of *Staphylococcus aureus* Strains Associated with Food Poisoning in Shenzhen, China

Xiaomei Yan[*], Bing Wang[*], Xiaoxia Tao, Qinghua Hu, Zhigang Cui, Jianzhong Zhang[#], Yiman Lin, Yuanhai, You, Xiaolu Shi, Hajo Grundmann

[*]These authors contributed equally to this work.

Published in the *Appl. Environ. Microbiol* (2012) 78, 6637-42

Abstract

To characterize the isolates of *Staphylococcus aureus* that were associated with staphylococcal food poisoning between 2006 and 2009 in Shenzhen, Southern China, a total of 52 *Staphylococcus aureus* isolates from 11 outbreaks were analyzed using multilocus sequence typing (MLST), *spa* typing, and pulsed-field gel electrophoresis (PFGE). PCR analysis was used to analyze the staphylococcal enterotoxin (*se*) genes *sea* to *sei*, and antimicrobial susceptibility testing was also performed. ST6 was the most dominant ST type, constituting 63.5% (34/52) of all of the isolates in 7 outbreaks. The next most common ST type was ST943, which constituted 23.1% (12/52) of the isolates that were collected from 3 outbreaks. T701, t091, and t2360 were the most predominant *spa* types, constituting 67.3% (35/52) of the isolates that were collected from 11 outbreaks. Three PFGE types, type (A, B, and C) were the most frequently observed types, constituting 84.6% (44/52) of all of the isolates. The most frequent enterotoxin gene that we detected was *sea* (45/52, 86.5%). Four *se* gene profiles were observed, including *sea* (n=45), *sec-seh* (n=3), *seb* (n=2), and *seg-sei* (n=2). With respect to antibiotic resistance, penicillin resistance was the most common (96.2%, 50/52), followed by resistance to tetracycline (28.8%, 15/52). Approximately 30.8% (16/52) of the isolates were resistant to at least two antibiotics, and 7.7% (4/52) of the isolates were resistant to three or more drugs. The two predominant *S. aureus* lineages, (1) PFGE types A and B with ST type ST6 and (2) PFGE type C with ST type ST943, were identified in the outbreaks.

Introduction

Staphylococcal food poisoning (SFP) is a frequent cause of food-borne gastroenteritis worldwide (15, 18, 26, 34). Between 2008 and 2010, a total of 371 outbreaks of bacterial foodborne diseases were reported in China, involving 20,062 individuals and leading to 41 deaths. Ninety-four outbreaks of SFP were reported to the National Monitoring Network between 2003 and 2007, involving 2,223 individuals and leading to 1,186 hospitalizations. *S. aureus* was the fifth most frequently observed pathogen after *Vibrio parahaemolyticus*, *Bacillus cereus*, *Bacillus proteus,* and Salmonella (17). In Shenzhen, eleven outbreaks of SFP were reported to the local monitoring network between 2006 and 2009, representing the second most frequent cause of bacterial food poisoning after *Vibrio parahaemolyticus*. Because most SFP cases are mild, the actual number of SFP cases is expected to be much higher than is reported (18).

SFP is associated with the toxinogenic *S. aureus* strains that express one or more of a family of genes that code for heat-stable enterotoxins (1). These genes share a common genetic relationship, structure, and function, and have a high degree of sequence homology (1). In addition to functioning as potent gastrointestinal toxins, staphylococcal enterotoxins (SE) also act as superantigens that stimulate non-specific T-cell proliferation, which can potentially cause toxic shock (1). A standard nomenclature was proposed such that only toxins that induce emesis following oral administration in a primate model are designated as SEs. Otherwise, the toxins are referred to as staphylococcal enterotoxin-like superantigens (SAgs) (16). In addition to the five classical types of SEs (SEA through SEE), sixteen more recently described SEs or SE-like toxins (SEG through SEV) have been described (15, 20, 22, 29, 30).

To understand the epidemiology, population biology and genetic diversity of enterotoxinogenic *S. aureus*, we employed the typing methods that have been previously been used to characterize hospital- and community-acquired *S.aureus* infections. To our knowledge, there have been few molecular epidemiologic investigations of *S.aureus*-associated SFP in China. The aim of this study was to use molecular epidemiology to both characterize *S. aureus*-associated SFP and to improve our understanding of the genetic relatedness of the more pathogenic strains. These results may provide insight into the spread of the isolates that are associated with outbreaks and may ultimately improve the control of SPF in Southern China.

MATERIALS AND METHODS

The isolates and the patient data were collected between 2006 and 2009 from 11 outbreaks that were reported in Shenzhen, Guangdong Province, Southern China. All of the isolates were identified at the species level by coagulase production using the Slidex Staph Plus kit (Murex Biotech, Kent, France) and PCR for the *nuc* gene (2). The SFP diagnosis was confirmed by any of the following: (i) the detection of SEs in leftover food, (ii) the isolation of *S.aureus* with the same enterotoxin type from both food and patients, and (iii) the isolation of *S.aureus* with the same enterotoxin type from different patients. An outbreak was defined by the identification of more than two epidemiologically associated cases.

Molecular typing

All of the isolates were characterized using pulsed-field gel electrophoresis (PFGE) and *spa* typing. Multilocus sequence typing was performed for eight isolates that included representatives of each *spa* type. PFGE was performed using the CHEF-DR III System (Bio-Rad, United States), as described previously (37). The digital images were analyzed by BioNumerics software (v. 5.10, Applied Maths) using the Dice coefficient and were generated by UPGMA with 1.5% tolerance and 1% optimization settings. A similarity cutoff of 80% and a difference of 6 bands were used to define a cluster, as described by Tenover (28). The isolates that exhibited identical or related PFGE patterns were considered to belong to the same clone. The clones were labeled with capital letters (A, B, C), and related profiles were indicated by adding a number (A1, A2, B1, B2, etc.).

The *Spa* and the MLST typing were performed as previously described (7, 10). Based Upon Repeat Pattern (BURP) analysis was used to cluster *spa* clonal complex (*spa*-CC) the spa types (19).

Identification of the SE genes using PCR

The genomic DNA was extracted using the DNeasy blood and tissue kit (Qiagen GmbH, Hilden, Germany). The amplifications were performed using a Mycycler^{TM} thermal cycler (Bio-Rad, Hercules, California, United States). The *sea, seb, sec, sed, see, seg, seh,* and *sei* genes were detected according to the methodology of previous studies (24, 25).

Antimicrobial susceptibility

A total of 18 antimicrobial agents were tested, including penicillin G, cefoxitin, oxacillin, piperacillin-tazobactam, ampicillin-sulbactam, cefazolin, vancomycin, teicoplanin, clindamycin, erythromycin, tetracycline, minocycline, ciprofloxacin, chloramphenicol, rifampin, gentamicin, trimethoprim-sulfamethoxazole and quinupristin-dalfopristin. All of the

antimicrobial agents were tested using disk diffusion (OXOID, Basingstoke, England). The CLSI zone diameter breakpoints were used to interpret the antimicrobial susceptibility of the analyzed strains.

Results

Epidemiological data and isolates

A total of 11 food poisoning outbreaks were reported between Jan 10, 2006 and Aug 24, 2009 (Table 1). Seventy-nine individuals were reported to be ill and suffered from abdominal pain (n=64), diarrhea (n=62), nausea (n=55), vomiting (n=45), giddiness (n=24) and headache (n=15). Seven outbreaks occurred in private households, two outbreaks occurred at dining halls, and the other two outbreaks occurred at a restaurant and a supermarket. The incubation period ranged from 1.5 to 11.5 hours. The *S. aureus* isolates that produced SEs were isolated from the food leftovers in nine outbreaks and were isolated from the patients and from the environment in another two outbreaks. A total of 52 *S. aureus* isolates were isolated from food (n=27), rectal swabs (n=15), feces (n=5), the environment (n=4) or the hand swab of a food handler (n=1) (Table 2). Twenty-six isolates were collected in 2006, twenty-three isolates were collected in 2007, two isolates were collected in 2008 and one isolate was collected in 2009.

MLST, *spa* and PFGE

Multilocus sequence typing for each of the identified *spa* types revealed five ST types, namely ST1, ST5, ST6, ST188 and ST943 (Table 2). ST6 was the most dominant ST type that was observed and was identified in 34 (63.5%) of the isolates from 7 outbreaks. Twelve isolates from 3 outbreaks (23.1%) were found to be ST943. *Spa* typing of all of the isolates yielded eight *spa* types and 1 *spa*-CC (Table 2). T701, t091 and t2360 were the most predominant *spa* types, constituting 67.3% (35/52) of all of the isolates in 11 outbreaks. The fifty-two isolates were also typed using PFGE (Table 2). The genetic analysis revealed ten different PFGE banding patterns (A through G) and six clusters (1 through 6) (Figure 1). Three PFGE types (A, B, and C) were the dominant types, constituting 84.6% (44/52) of all of the isolates.

Table 1. Epidemiological data from the 12 food poisoning outbreaks in Shenzhen city

Outbreak	Date (month- day -year)	Number of ill/ hospitalized/ death/ at risk	Site	Incubation period (median)	Symptoms (Number)[a]	Food	SFPO assessment[b]
1	Jan 10, 2006	16/13/0/200	dining hall	1.5-9.5 h (5)	N（15）、V（13）、D（9）、AP（9）、H（2）、G（5）	bean curd, cooked duck, cooked meat, potato chips, cooked spareribs	C
2	Aug 11, 2006	5/5/0/10	private household	1.5-5 h (3.5)	N（5）、V（2）、D（4）、AP（4）、H（2）、G（1）	chicken bittern duck sausage， cooked squid head	C
3	Aug 28, 2006	3/3/0/8	private household	1.5-3 h (2)	N（3）、V（2）、D（3）、AP（3）、H（1）、G（1）	none[c]	C
4	Sep 15, 2006	3/3/0/5	private household	1.5-3 h (2)	N（1）、V（2）、D（3）、AP（3）、H（1）、G（1）	cake and dried meat floss cake	C
5	Nov 15, 2006	5/5/0/6	private household	2-5 h (3)	N（3）、V（3）、D（4）、AP（5）、H（1）、G（1）	raw peppery radish	**C**
6	Feb 20, 2007	3/3/0/3	private	1.5-3.5 h (2.3)	N（3）、V（3）、D（3）、	cured meat and peanut	C

			household		AP（3）、H（2）、G（2）		
7	Sep 11, 2007	5/5/0/5	private household	1.5-5 h (3)	N（2）、V（2）、D（5）、AP（5）、H（1）、G（1）	bread	**C**
8	Sep 18, 2007	5/5/0/10	private household	1.5-5 h (3.5)	N（3）、V（2）、D（5）、AP（5）、H（1）、G（1）	steamed twisted roll, bittern chicken wing, roasted chicken and sausage	**C**
9	Dec 20, 2007	3/3/0/4	restaurant	1.5-3 h (2)	N（1）、V（1）、D（3）、AP（3）	food	**C**
10	Aug 14, 2008	3/3/0/3	supermarket	7.5-11.5 h (9.8)	N（3）、V（3）、D（3）、AP（3）、H（1）、G（1）	raw kelp	C
11	Aug 24, 2009	28/28/0/1500	dining hall	1.5-6.6 h (3.5)	N（11）、V（6）、D（17）、AP（19）、H（0）、G（7）	none[c]	S

[a] V, vomiting; D, diarrhea; AP, abdominal pain; N, nausea; H, headache; G, giddiness

[b] SFP outbreak assessment based on SFP diagnosis and outbreak definition. confirmed; S, suspected

[c] *Staphylococcus aureus* was not isolated from food but from the patients.

Table 2. Analysis of the *Staphylococcus aureus* isolates from the food poisoning outbreaks in Shenzhen, China

Outbreak	Origin (no. of isolates)[a]	MLST	*Spa* (CCs)	PFGE pattern [b]	Toxin genes [c]	Resistance to [d]
1	fd (3)	6	t701 (701)	A1	*sea*	PEN, TC
	fd (6)	943	t091 (singleton)	C	*sea*	PEN, TC/PEN
	rs (1)	943	t091 (singleton)	C	*sea*	PEN, EM
2	fd (3)	943	t091 (singleton)	C	*sea*	PEN/PEN, CM, TC
	rs (2)	6	t701 (701)	A1	*sea*	PEN, TC
	en (1)	943	t091 (singleton)	C	*sea*	PEN, TC
3	rs (1), en (1)	1	t127 (singleton)	F	*sec, seh*	PEN,CM,EM,TC,RI
	rs (1)	1	t127 (singleton)	F	*sec, seh*	PEN, CM, EM, TC
4	fd (2)	6	t5777(701)	A1	*sea*	PEN/PEN, TC
	fc (1)	6	t5777(701)	B1	*sea*	PEN
5	fd (1), rs (3)	6	t701 (701)	B2	*sea*	PEN
6	fd (2), fc (3), en (2)	6	t5593(701)	A1	*sea*	PEN
7	fd (2)	5	t954 (singleton)	G1/G2	*seg, sei*	Susceptible
8	fd (5), rs (6)	6	t2360(701)	B1	*sea*	PEN
9	fd (2), rs (1)	6	t701 (701)	A2	*sea*	PEN
10	fd(1), fhs(1)	188	t189	E	*seb*	PEN

11	fc(1)	943	(singleton) t091	D	*sea*	PEN, TC

[a] A total of 52 *S. aureus* isolates were identified. The origin of the samples included food (27), rectal swabs (15), feces (5), the environment (4), and the food handler (1). fd, food; fc, feces; rs, rectal swab; en, environment; fhs, food handler hand swab.

[b] The pulsed-field gel electrophoresis (PFGE) types are alphabetically designated, and the subtypes are numerically designated.

[c] Tested for the *sea, seb, sec, sed, see, seg, seh, sei* genes.

[d] PEN, Penicillin; TC, Tetracycline; EM, Erythromycin; CM, Clindamycin; RI, Rifampin

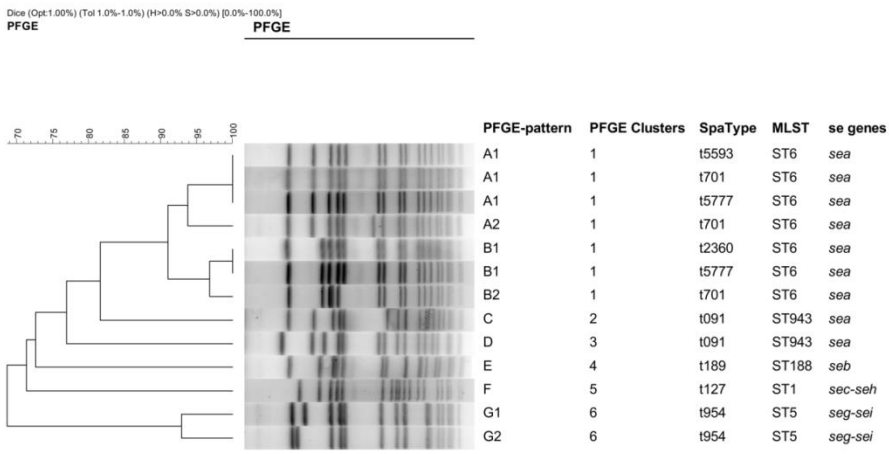

Fig.1. Dendrogram of the pulsed-field electrophoresis gel (PFGE) patterns of the *S.aureus* strains that were associated with SPF cases between 2006 and 2009.

There were two outbreaks (1 and 2) for which two ST, *spa* and PFGE types were observed in the same outbreak, namely ST6: t701: A1 and ST943: t091: C (ST: *spa*: PFGE). A very similar clone to ST943: t091: D was also identified in outbreak 11. Outbreaks 5 and 9 exhibited the same ST and *spa* types (ST6: t701) but exhibited different PFGE types (PFGE B2 and A2). The ST6 type was also identified in outbreaks 4, 6 and 8, with different *spa* and PFGE types (t5777: A1/B1, t5593: A1, and t2360: B1, respectively). The ST1: t127: F, ST5: t954: G1/G2 and ST188: t189: E strains were only observed in outbreak 3, 7 and 10, respectively.

Exotoxin genes

The most frequently identified enterotoxin gene was *sea* (45/52, 86.5%), followed by *sec* (4/52, 7.7%), *seb* (2/52, 3.8%), *seh* (3/52, 5.8%), *seg* (2/52, 3.8%) and *sei* (2/52, 3.8%). Four *se* gene profiles were observed, namely *sea* (n=45), *sec-seh* (n=3), *seb* (n=2), *seg-sei* (n=2). Seven outbreaks (1, 2, 4, 5, 6, 8 and 10) with the *sea* gene profile were identified. Outbreaks 3 (*sec-she*) and 7 (*seg-sei*) occurred in 2006 and 2007, each. The PFGE patterns were strongly correlated with *se* gene profiles (Figure 1).

Antibiotic resistance

Only two strains were observed to be susceptible to all of the drugs that were tested in this study (Table 2). No MRSA strains were detected. Penicillin resistance was the most commonly observed resistance (96.2%, 50/52) of the tested strains, followed by tetracycline resistance (28.8%, 15/52). Erythromycin and clindamycin resistance were observed less frequently (7.7%, 4/52). Two strains were found to be resistant to rifampin. Sixty-five percent of all of the isolates (34/52) were only resistant to PEN, followed by resistance to PEN-TC (11/52, 21.2%). Approximately 30.8% (16/52) of the isolates were resistant to at least two antibiotics, and four strains were resistant to three or more drugs.

DISCUSSION

In this study, the two predominant *S. aureus* lineages were identified, corresponding to (1) PFGE types A and B with the ST type ST6 and (2) PFGE type C with the ST type ST943. To our knowledge, this study is the first description of the genetic diversity of *S. aureus* isolates that have been associated with the food poisoning outbreaks that occurred between 2006 and 2009 in Shenzhen, Southern China.

Based on our results, of the five sequence types (ST1, ST5, ST6, ST188 and ST943), ST6 was the most dominant clone in Shenzhen, China in this time period. T701 (ST6) and t189 (ST188) were also observed among the SFP isolates in the isolates from two outbreaks in Ma'anshan, Anhui Province (33). However, in South Korea, the ST1, ST59, and ST30 strains were the clones that were most frequently associated with SFP (3). Within the MLST database, prior to 2004, all of the isolates with the ST type ST6 were methicillin-susceptible *Staphylococcus aureus* (MSSA) from Australia, the United Kingdom, Thailand, Japan and Gambia. The ST6 MRSA strain has rarely been isolated, but community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) clones were reported in Kuwait hospitals (32), Japan (12) and Lebanon (31). According to previous research, ST6 is not the predominant *S.aureus* lineage that is observed in hospitals and animals in China (6, 8, 9, 35, 36, 38, 40, 41). Why ST6 MSSA became the dominant ST type that causes SFP and whether

ST6 was the unique ST type in food products or among SFP isolates in China should be investigated further.

Based on phylogenetic inference, the outbreaks that were analyzed in this study were caused by several similar clones (Fig 2). The ST6-t701-A1 clone had the same ST and *spa* types as did the ST6-T701-A2 and ST6-t701-B2 clones. The PFGE pattern indicated several bands that differ between these strains, suggesting certain large-scale changes in the accessory genome. The same situation was observed with respect to the ST943-t091-C and ST943-t091-D clones. Phylogenetic inference suggests that ST6-t701-A1/A2/B2 are the ancestors of ST6-t5777-A1/B1 and ST6-t2360-B1, which separately acquired two spa repeats (r24 and r25) or clonally related PFGE subtype changes. Based on BURP analysis results, ST6-t701-A1 is also the ancestor of ST6-t5593-A1, which acquired one spa repeat (r19) and lost another (r25). This suggested temporal relationship should be confirmed by studies that include the analysis of additional isolates. Further studies are required to elucidate the transmission routes of *S.aureus* strains that are associated with SFP and to provide the tools that should be used to prevent the spread of SFP.

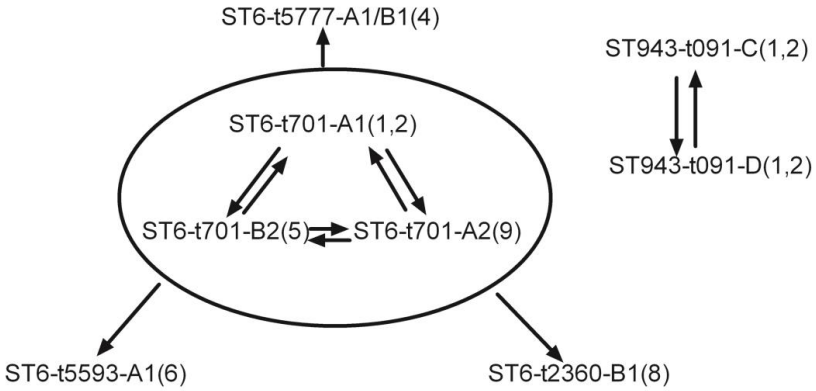

Fig. 2. Phylogenetic inference of the different *S.aureus* clones that were examined in this study: The model was based on ST types, BURP analysis and PFGE types. The arrows indicate the directions of the changes between clones. The clone name is designated in the following way: MLST-*spa*-PFGE (outbreak).

PFGE, *spa* and MLST typing were used for the molecular epidemiological investigation of the isolates in this study. PFGE is a technique that is still widely used for *S.aureus* isolate

typing, primarily because of its excellent discriminatory power, especially with respect to the analysis of short-term epidemiology, despite the difficulty when comparing the results obtained in different laboratories (21). Our results revealed 10 patterns that can be classified by PFGE and 8 types that can be classified by *spa* typing. Based on PFGE analysis, the PFGE pattern of the outbreak strains were identical within each outbreak, except for outbreaks 1 and 2, which were caused by a mixed clones. In two outbreaks (4 and 7), two similar PFGE patterns were detected in each outbreak, whereas the *spa* and MLST types remained indistinguishable. PFGE was more effective than sequencing with respect to identification capability. However, there were three *spa* types (t701, t5593, and t5777) from three outbreaks with the same PFGE type (A1). Two *spa* types (t2360 and t5777) were also detected in the two outbreaks that were correlated with the PFGE type B1. We could not identify any epidemiological relationship between these outbreaks. According to the *spa* repeat analysis, the strains that shared the same PFGE type were very closely related, indicating that these strains belong to the same clone and differed only as a result of the recombination of certain genes; these strains were indistinguishable based on PFGE analysis. This study highlights the fact that PFGE typing is effective in describing strain population structure but, due to the oligoclonality of SFP outbreaks, is limited in its epidemiological resolution.

The data in this study revealed that the *sea* gene is dominant in the *S.aureus* isolates that are associated with food poisoning in Shenzhen, China. In fact, the staphylococcal enterotoxin type that is most frequently involved in food poisoning outbreaks worldwide is SEA, which is associated with other staphylococcal enterotoxins (1, 3, 5, 13, 26, 39). The SFP that is caused by *sea* alone maybe unique in Shenzhen. In this study, an outbreak with *seg-sei* profile was observed in a single family in 2007. To our knowledge, this is the first report of two *S.aureus* strains with the *seg-sei* profile that has been associated with food poisoning in China. The two strains with the closely related PFGE types G1 and G2 were ST5- *spa* t954 clones, which differed from the *seg-sei* positive strains that were observed in Korea, which carried the ST type ST20. The *seg* and *sei* genes were originally identified in two separate strains (20). The coexistence of *seg* and *sei* was unsurprising, together with *sem, sen*, and *seo.* All of these five genes belong to an enterotoxin gene cluster (*egc*), which is located on a genomic island; the detection of one of these genes generally indicates the presence of others (11, 14). Although the involvement of more recently identified SEs in SFP is not yet fully understood, this factor is important for the long-term monitoring of the changing epidemiology of SE and SE-encoding genes in cases of SFP.

Compared with the MRSA clones that are principally responsible for hospital- and community-acquired infections, MSSA lineages that are associated with the SFP outbreaks were more susceptible to antibiotics (35). The majority of the isolates that were examined in this study are only resistant to one or two antibiotics. The observed high resistance rates to penicillin concurred with several previous studies of *S.aureus* isolates from food products in both China and other countries (4, 23). However, with respect to tetracycline resistance, the previously reported rates varied greatly (4,13). We also detected three multidrug resistant isolates, which were isolated from two patients and the environment. These isolates were ST1: t127: F and exhibited *sec-seh* enterotoxin profiles and were detected in isolates from outbreak 3, which occurred in 2006. The incidence of antimicrobial resistance in human infections is directly related to the prevalence of resistant bacteria in food products (27); antibiotic resistance to SPF case isolates must therefore be considered.

It will be necessary to better understand the population structure of MSSA carriers and clinical isolates to determine if *S.aureus* strains that cause SFP represent different lineages from those that are commonly carried and from those that cause pyogenic infections.

Acknowledgements

This work was supported by "major infectious diseases such as AIDS and viral hepatitis prevention and control" technology major projects (2008ZX10004-002 and 2008ZX10004-008) from the Ministry of Science and Technology, the People's Republic of China and the Shenzhen Municipal Research Fund (201102103).

Reference List

1. Balaban, N. and A. Rasooly. 2000. Staphylococcal enterotoxins. Int.J.Food Microbiol. 61:1-10.
2. Brakstad, O. G., K. Aasbakk, and J. A. Maeland. 1992. Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the nuc gene. J.Clin.Microbiol. 30:1654-60.
3. Cha, J. O., J. K. Lee, Y. H. Jung, J. I. Yoo, Y. K. Park, B. S. Kim, and Y. S. Lee. 2006. Molecular analysis of *Staphylococcus aureus* isolates associated with staphylococcal food poisoning in South Korea. J.Appl.Microbiol. 101:864-71.
4. Chao, G., X. Zhou, X. Jiao, X. Qian, and L. Xu. 2007. Prevalence and antimicrobial resistance of foodborne pathogens isolated from food products in China. Foodborne.Pathog.Dis. 4:277-84.
5. Chen, Z., Jiang Y., Xiong, Y., Zhou, S., Long, Y. 2009. Detection of toxin genes among *Staphylococcus aureus* isolates from food - poisoning episodes by PCR Assay. Chinese Journal of Health Laboratory Technology.19: 21-3. (In Chinese.)
6. Cui, S., J. Li, C. Hu, S. Jin, F. Li, Y. Guo, L. Ran, and Y. Ma. 2009. Isolation and characterization of methicillin-resistant *Staphylococcus aureus* from swine and workers in China. J.Antimicrob.Chemother. 64:680-3.
7. Enright, M. C., N. P. Day, C. E. Davies, S. J. Peacock, and B. G. Spratt. 2000. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. J.Clin.Microbiol. 38:1008-15.
8. Geng, W., Y. Yang, D. Wu, G. Huang, C. Wang, L. Deng, Y. Zheng, Z. Fu, C. Li, Y. Shang, C. Zhao, S. Yu, and X. Shen. 2010. Molecular characteristics of community-acquired, methicillin-resistant *Staphylococcus aureus* isolated from Chinese children. FEMS Immunol.Med.Microbiol. 58:356-62.
9. Geng, W., Y. Yang, D. Wu, W. Zhang, C. Wang, Y. Shang, Y. Zheng, L. Deng, Z. Fu, X. Li, S. Yu, and X. Shen. 2010. Community-acquired, methicillin-resistant *Staphylococcus aureus* isolated from children with community-onset pneumonia in China. Pediatr.Pulmonol. 45:387-94.
10. Harmsen, D., H. Claus, W. Witte, J. Rothganger, H. Claus, D. Turnwald, and U. Vogel. 2003. Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for *spa* repeat determination and database management. J.Clin.Microbiol. 41:5442-8.
11. Jarraud, S., M. A. Peyrat, A. Lim, A. Tristan, M. Bes, C. Mougel, J. Etienne, F. Vandenesch, M. Bonneville, and G. Lina. 2001. *egc*, a highly prevalent operon of enterotoxin gene, forms a putative nursery of superantigens in *Staphylococcus aureus*. J.Immunol. 166:669-77.
12. Kawaguchiya, M., N. Urushibara, O. Kuwahara, M. Ito, K. Mise, and N. Kobayashi. 2011. Molecular characteristics of community-acquired methicillin-resistant *Staphylococcus aureus* in Hokkaido, northern main island of Japan: identification of sequence types 6 and 59 Panton-Valentine leucocidin-positive community-acquired methicillin-resistant *Staphylococcus aureus*. Microb.Drug Resist. 17:241-50.
13. Kerouanton, A., J. A. Hennekinne, C. Letertre, L. Petit, O. Chesneau, A. Brisabois, and M. L. De Buyser. 2007. Characterization of *Staphylococcus aureus* strains associated with food poisoning outbreaks in France. Int.J.Food Microbiol. 115:369-75.
14. Kuroda, M., T. Ohta, I. Uchiyama, T. Baba, H. Yuzawa, I. Kobayashi, L. Cui, A. Oguchi, K. Aoki, Y. Nagai, J. Lian, T. Ito, M. Kanamori, H. Matsumaru, A. Maruyama, H. Murakami, A. Hosoyama, Y. Mizutani-Ui, N. K. Takahashi, T. Sawano, R. Inoue, C. Kaito, K. Sekimizu, H. Hirakawa, S. Kuhara, S. Goto, J. Yabuzaki, M. Kanehisa, A.

Yamashita, K. Oshima, K. Furuya, C. Yoshino, T. Shiba, M. Hattori, N. Ogasawara, H. Hayashi, and K. Hiramatsu. 2001. Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*. Lancet. 357:1225-40.

15. Le, L. Y., F. Baron, and M. Gautier. 2003. *Staphylococcus aureus* and food poisoning. Genet.Mol.Res. 2:63-76.
16. Lina, G., G. A. Bohach, S. P. Nair, K. Hiramatsu, E. Jouvin-Marche, and R. Mariuzza. 2004. Standard nomenclature for the superantigens expressed by Staphylococcus. J.Infect.Dis. 189:2334-6.
17. Mao, X., Hu J., Liu X. 2010. Epidemiological Characteristics of Bacterial Foodborne Disease During the Year 2003- 2007 in China. Chinese Journal of Food Hygiene. 22: 224-8. (In Chinese.)
18. Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe. 1999. Food-related illness and death in the United States. Emerg.Infect.Dis. 5:607-25.
19. Mellmann A, Weniger T, Berssenbrugge C, Rothganger J, Sammeth M, Stoye J and Harmsen D. 2007. Based Upon Repeat Pattern (BURP): an algorithm to characterize the long-term evolution of *Staphylococcus aureus* population based on *spa* polymorphisms.BMC Microbiol. 7:98.
20. Munson, S. H., M. T. Tremaine, M. J. Betley, and R. A. Welch. 1998. Identification and characterization of staphylococcal enterotoxin types G and I from *Staphylococcus aureus*. Infect.Immun. 66:3337-48.
21. Murchan, S., M. E. Kaufmann, A. Deplano, R. R. de, M. Struelens, C. E. Zinn, V. Fussing, S. Salmenlinna, J. Vuopio-Varkila, S. N. El, C. Cuny, W. Witte, P. T. Tassios, N. Legakis, L. W. van, B. A. van, A. Vindel, I. Laconcha, J. Garaizar, S. Haeggman, B. Olsson-Liljequist, U. Ransjo, G. Coombes, and B. Cookson. 2003. Harmonization of pulsed-field gel electrophoresis protocols for epidemiological typing of strains of methicillin-resistant *Staphylococcus aureus*: a single approach developed by consensus in 10 European laboratories and its application for tracing the spread of related strains. J.Clin.Microbiol. 41:1574-85.
22. Ono, H. K., K. Omoe, K. Imanishi, Y. Iwakabe, D. L. Hu, H. Kato, N. Saito, A. Nakane, T. Uchiyama, and K. Shinagawa. 2008. Identification and characterization of two novel staphylococcal enterotoxins, types S and T. Infect.Immun. 76:4999-500
23. Pereira, V., C. Lopes, A. Castro, J. Silva, P. Gibbs, and P. Teixeira. 2009. Characterization for enterotoxin production, virulence factors, and antibiotic susceptibility of *Staphylococcus aureus* isolates from various foods in Portugal. Food Microbiol. 26:278-82.
24. Rosec, J. P. and O. Gigaud. 2002. Staphylococcal enterotoxin genes of classical and new types detected by PCR in France. Int.J.Food Microbiol. 77:61-70.
25. Sergeev, N., D. Volokhov, V. Chizhikov, and A. Rasooly. 2004. Simultaneous analysis of multiple staphylococcal enterotoxin genes by an oligonucleotide microarray assay. J.Clin.Microbiol. 42:2134-43.
26. Shimizu, A., M. Fujita, H. Igarashi, M. Takagi, N. Nagase, A. Sasaki, and J. Kawano. 2000. Characterization of *Staphylococcus aureus* coagulase type VII isolates from staphylococcal food poisoning outbreaks (1980-1995) in Tokyo, Japan, by pulsed-field gel electrophoresis. J.Clin.Microbiol. 38:3746-9.
27. Smith, D. L., A. D. Harris, J. A. Johnson, E. K. Silbergeld, and J. G. Morris, Jr. 2002. Animal antibiotic use has an early but important impact on the emergence of antibiotic resistance in human commensal bacteria. Proc.Natl.Acad.Sci.U.S.A. 99:6434-9.
28. Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J.Clin.Microbiol. 33:2233-9.

29. Thomas, D., S. Chou, O. Dauwalder, and G. Lina. 2007. Diversity in *Staphylococcus aureus* enterotoxins. Chem.Immunol.Allergy. 93:24-41.
30. Thomas, D. Y., S. Jarraud, B. Lemercier, G. Cozon, K. Echasserieau, J. Etienne, M. L. Gougeon, G. Lina, and F. Vandenesch. 2006. Staphylococcal enterotoxin-like toxins U2 and V, two new staphylococcal superantigens arising from recombination within the enterotoxin gene cluster. Infect.Immun. 74:4724-34.
31. Tokajian, S. T., P. A. Khalil, D. Jabbour, M. Rizk, M. J. Farah, F. A. Hashwa, and G. F. Araj. 2010. Molecular characterization of *Staphylococcus aureus* in Lebanon. Epidemiol.Infect. 138:707-12.
32. Udo, E. E. and E. Sarkhoo. 2010. The dissemination of ST80-SCCmec-IV community-associated methicillin resistant *Staphylococcus aureus* clone in Kuwait hospitals. Ann.Clin.Microbiol.Antimicrob. 9:31.
33. Y.WANG，D.WANG，S.ZHAN，J.ZHENG，Y.TAO，L.WANG，X.YAN，X.TAO，J.ZHANG，B.KAN. 2011. Identification of *Staphylococcus aureus* and SpA polymorphisms in Ma′ anshan City. J of Pub Health and Prev Med. Vol. 22:50-3.
34. Wieneke, A. A., D. Roberts, and R. J. Gilbert. 1993. Staphylococcal food poisoning in the United Kingdom, 1969-90. Epidemiol.Infect. 110:519-31.
35. Wu, D., Q. Wang, Y. Yang, W. Geng, Q. Wang, S. Yu, K. Yao, L. Yuan, and X. Shen. 2010. Epidemiology and molecular characteristics of community-associated methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* from skin/soft tissue infections in a children's hospital in Beijing, China. Diagn.Microbiol.Infect.Dis. 67:1-8.
36. Xiao, Y. H., C. G. Giske, Z. Q. Wei, P. Shen, A. Heddini, and L. J. Li. 2011. Epidemiology and characteristics of antimicrobial resistance in China. Drug Resist.Updat. 14:236-50.
37. Yan, X., X. Tao, L. He, Z. Cui, and J. Zhang. 2011. Increasing resistance in multiresistant methicillin-resistant *Staphylococcus aureus* clones isolated from a Chinese hospital over a 5-year period. Microb.Drug Resist. 17:235-39.
38. Yao, D., F. Y. Yu, Z. Q. Qin, C. Chen, S. S. He, Z. Q. Chen, X. Q. Zhang, and L. X. Wang. 2010. Molecular characterization of *Staphylococcus aureus* isolates causing skin and soft tissue infections (SSTIs). BMC.Infect.Dis. 10:133.
39. Zhang, Li., LI, Y., MA, X. 2009.Research of enterotoxin and its gene in *Staphylococcus aureus* strains isolated from food poisoning and food samples. Chinese Journal of Health Laboratory Technology.19:2851-3. (In Chinese.)
40. Zhang, W., Z. Hao, Y. Wang, X. Cao, C. M. Logue, B. Wang, J. Yang, J. Shen, and C. Wu. 2011. Molecular characterization of methicillin-resistant *Staphylococcus aureus* strains from pet animals and veterinary staff in China. Vet.J. 190:e125-9.
41. Zhang, W., X. Shen, H. Zhang, C. Wang, Q. Deng, L. Liu, and Y. Yang. 2009. Molecular epidemiological analysis of methicillin-resistant *Staphylococcus aureus* isolates from Chinese pediatric patients. Eur.J.Clin.Microbiol.Infect.Dis. 28:861-4.

Chapter 4

Staphylococcus aureus ST398 from Slaughter Pigs in Northeast China

Xiaomei Yan[#], Xiaojie Yu[#], Xiaoxia Tao, Jianfeng Zhang, Binghua Zhang, Rui Dong, Chengyu Xue, Hajo Grundmann[*], Jianzhong Zhang[*]

[#] These authors contributed equally to this work.

Published in the *Int J Med Microbiol* (2014) 304: 379–83

Abstract

To describe the prevalence and population structure of *Staphylococcus aureus* bacteria that colonize pigs at slaughterhouses in northeastern China, nose swabs were collected from pigs in two slaughterhouses in Harbin, Heilongjiang Province, China in 2009. *S. aureus* isolates were characterized by multilocus sequence typing (MLST), *spa* typing, SCC*mec* typing, antimicrobial susceptibility testing and *pvl* gene detection. A total of 200 *S. aureus* isolates were collected from 590 pigs (33.9%, 200/590), of which 162 (81%, 162/200) were methicillin-susceptible *S. aureus* (MSSA) and 38 (19%, 38/200) were methicillin-resistant *S. aureus* (MRSA). Ninety-nine of the MSSA isolates (99/162, 61.1%) were ST398, which represented the dominant sequence type overall. Eighty-seven isolates were ST9 (87/200, 43.5%), and all MRSA belonged to that sequence type which consisted of the *spa* types t899 and t2922. Among the MSSA strains, t034, t899 and t4358 were the most dominant *spa* types (139/162, 85.8%). All MRSA isolates harbored SCC*mec* type IVb. The *pvl* gene was only detected in 3 ST7/t2119 MSSA isolates. All MRSA but more importantly also 82.7% (134/162) of the MSSA isolates were resistant to six or more antibiotics. Moreover, a novel resistance determinant-*lsa(E)* was identified among 22% (44/200) of all isolates. In conclusion, pigs in northeast China are frequently colonized with ST398 MSSA. MRSA with this sequence type, typically associated with pigs in Europe, was not found. High levels of multiple antibiotic resistance among MRSA isolates as well as MSSA isolates are a public health concern.

Introduction

Staphylococcus aureus is a well-known commensal and pathogen of many animal species, including humans. Although most *S. aureus* are host-specific, the potential for animals to act as a source of *S. aureus* infections for humans has been shown for some clonal lineages, such as sequence type (ST) 398 (Garcia-Alvarez et al., 2011; van Cleef et al., 2011). Moreover, the propensity of *S. aureus* to develop multiple drug resistance (MDR) has played the key role in triggering pandemic spread (Holden et al., 2013). Hence, farm animals could be an important ecological niche for the emergence of MDR *S. aureus* because massive antibiotics use for treatment, prevention of diseases or growth promotion provides the necessary evolutionary constraints.

Methicillin-resistant *S. aureus* (MRSA) in pigs was first reported in France and was subsequently demonstrated to belong to a distinct clone, ST398 (Armand-Lefevre et al., 2005). Later, the ST398 MRSA clone was discovered to be widespread in pig farms in the Netherlands, where transmission to humans was reported for the first time (Voss et al., 2005; Armand-Lefevre et al., 2005). ST398 MRSA has been widely identified in several countries in Europe (Witte et al., 2007), North America (Smith et al., 2009; Khanna et al., 2008) and Asia (Lim et al., 2012; Sergio et al., 2007). ST398 MRSA possess several typical features, including (i) non-typability by standard *SmaI* pulsed-field gel electrophoresis analysis due to DNA methylation, (ii) t034, t011, and t108 being the dominant *spa* types, (iii) presence of SCC*mec* types IVa or V, (iv) absence of *luk*S and *luk*F coding for PVL and (v) resistance to tetracycline (Vanderhaeghen et al., 2010; Bens et al., 2006). Persons in direct contact with MRSA-positive animals have an increased risk of becoming MRSA positive (Morgan, 2008).

In China, previous studies revealed ST9 MRSA isolates as a predominant clone in pigs (Wagenaar et al., 2009; Cui et al., 2009), whereas ST398-MRSA-V-*spa* t034 was only reported once, in a pet hospital in Beijing (Zhang et al., 2011), and was likely of human origin. Little is known about the prevalence, population structure and antibiotic susceptibility of methicillin-susceptible *S. aureus* (MSSA) carried by pigs in China. The objective of this study was to characterize the prevalence of *S. aureus* and its population structure among colonized pigs in northeastern China.

Materials and Methods

Sample collection

From September through November 2009, nose swabs were taken from 590 pigs in two slaughterhouses located 68 km apart in Harbin city, Heilongjiang Province, northeastern

China. Pigs came to one slaughterhouse from 26 medium-sized industrial farms and to the other slaughterhouse from 29 small private farms. Pigs were slaughtered at the same day after they were collected from different farms. Samples from individual pigs were processed separately.

Sample preparation

Swabs were enriched in tryptic soy broth (OXOID, Basingstoke, England) with 7% NaCl at 37°C for 24 h and then plated onto mannitol salt agar (OXOID, Basingstoke, UK) and cultured at 37℃ for 24 h. Presumptive *S. aureus* colonies were identified at the species level by coagulase production using the Slidex Staph Plus kit (Murex Biotech, Kent, UK) and PCR for the *nuc* gene (Brakstad et al., 1992). The presence of the *mecA* gene was determined and used to define the MRSA genotype (Brakstad et al., 1992).

Molecular typing

All of the isolates were characterized using *spa* typing (Harmsen et al., 2003) and multilocus sequence typing (MLST) (Enright et al., 2000). Based Upon Repeat Pattern (BURP) analysis was used to group *spa* types into *spa* clonal complexes (*spa*-CCs) (Mellmann et al., 2007). The *spa* type was excluded from BURP analysis if it was shorter than 5 repeats. All MRSA isolates were investigated by SCC*mec* typing (Zhang et al., 2005).

Antimicrobial susceptibility

A total of 21 antimicrobial agents were tested, including penicillin (PEN), cefoxitin (FOX), tetracycline (TET), chlortetracycline (CTT), ciprofloxacin (CIP), chloramphenicol (CHL), erythromycin (ERY), clindamycin (CLI), gentamicin (GEN), rifampicin (RIF), trimethoprim-sulfamethoxazole (SXT), nitrofurantoin (NIT), quinupristin/dalfopristin (Q/D), vancomycin (VAN), teicoplanin (TEC), linezolid (LZD), tigecycline (TGC), daptomycin (DAP), fusidic acid (FUS), fosfomycin (FOF) and mupirocin (MUP). Eighteen antimicrobial agents were tested using the agar dilution method on Mueller-Hinton agar; the others (quinupristin/dalfopristin, tigecycline and daptomycin) were tested by Etest (AB bioMérieux, Solna, Sweden). The MICs for most of the antimicrobials were interpreted using CLSI criteria (Clinical and Laboratory Standards Institute, 2010), but the EUCAST clinical breakpoint (www.eucast.org) was used for fosfomycin and chlortetracycline. The resistance breakpoints for mupirocin and fusidic acid were defined as previously described (Finlay et al., 1997; MacGowan and Wise, 2001). *S. aureus* ATCC29213 was included for internal quality control. All quinupristin/dalfopristin resistant isolates were investigated for the resistance genes *vat*(A), *vat*(B), *vat*(C), *vga*(A), *vga*(A)*variant, vga*(B), *vga*(C), *vga*(E), *cfr* and *lsa*(E) by PCR

(Table S1).

***pvl* gene detection**

Panton-Valentine leukocidin (PVL) genes (*luk*S-PV and *luk*F-PV) were detected by PCR as previously described (Lina et al., 1999).

Statistical analysis

The quantitative variables were analyzed using chi-square tests in PASW Statistics 18.0.3 (SPSS Inc., Chicago, Illinois, US). P-values of ≤0.05 were considered statistically significant.

Results

Prevalence of *S. aureus*

A total of 200 *S. aureus* isolates were recovered from 590 slaughter pigs (200/590, 33.9%), including 38 MRSA isolates (38/590, 6.4%). The prevalence of MRSA among pigs and the number of MRSA-positive farms were significantly higher in Slaughterhouse 1 (Table 1).

Table 1. Prevalence of *Staphylococcus aureus* and Methicillin-resistant *S. aureus* (MRSA) among pigs from two slaughterhouses in northeast China during September–November 2009.

Slaughterhouse	No. of nasal swabs	No. of *S. aureus* (%)	No. of MRSA (%)	MRSA/*S.aureus* (%)	No. of farms with MRSA (%)
S1	313	104 (104/313, 33.2%)	31 (31/313, 9.9%)	29.8	14 (14/26, 53.8%)
S2	277	96 (96/277, 34.7%)	7 (7/277, 2.5%)	7.3	6 (6/29, 20.7%)
P-value	-	0.714	<0.0001	<0.0001	0.011

Molecular characterization of *S. aureus* isolates

MLST typing for all the isolates revealed seven ST types: ST7, ST9, ST398, ST2113, ST2366, ST1375 and ST2773 (Table 2). Among MSSA, ST398 was the most frequent ST type and was identified in 99 of 162 isolates (99/162, 61.1%); this was followed by ST9 (49/162, 30.2%). ST9 was the only ST type among the 38 MRSA isolates. ST2113, ST2366 and ST2773 were new ST types. ST2366 represents a single locus variant (SLV) of ST9, while ST1375 represents a SLV of ST398. *Spa* typing of all of the isolates identified nine *spa*

types and 2 *spa*-CCs (Table 2). The dominant *spa* type was t899 (33/38, 86.8%) among MRSA isolates, while t034 and t899 were the most prevalent *spa* types among MSSA, representing 55.6% (90/162) and 30.9% (50/162), respectively. Based on *spa* repeat and BURP analysis, two new *spa* types, t1446 and t5462, belonging to ST398 may have evolved

Table 2. Molecular characteristics of *Staphylococcus aureus* isolates from slaughter pigs

MLST types	SPA types (CCs)	MSSA/MRSA	Number of isolates	SCC*mec*	Number of PVL^+ isolates
ST398	t034 (034)	MSSA	90	-	0
	t1446 (034)	MSSA	7	-	0
	t5462 (034)	MSSA	3	-	0
ST9	t899 (899)	MSSA	37	-	0
	t4358 (899)	MSSA	12	-	0
	t4474 (899)	MSSA	1	-	0
	t899 (899)	MRSA	33	IVb	0
	t2922 (excluded)	MRSA	4	IVb	0
	NT	MRSA	1	IVb	0
ST2113	t6386 (singleton)	MSSA	2	-	0
ST7	t2119 (singleton)	MSSA	8	-	3
ST2366	NT	MSSA	1	-	0
Non-typable (NT)	NT	MSSA	1	-	0

in the MSSA isolates from t034 *spa* types that acquired one new *spa* repeat (r31) and three *spa* repeats (r34-r24-r25), respectively. In MRSA isolates, t899 is the probable ancestor of t4358 and t4474, which have separately acquired one *spa* repeat (r02) and changed one *spa* repeat from r07 to r26 by point mutation. All MRSA isolates were SCC*mec* type IVb and *pvl*

negative. Three MSSA isolates with ST7 were found to be *pvl* positive.

Antimicrobial susceptibility

All MRSA isolates were resistant to most of the antibiotics tested (Table 3). Importantly, two predominant multidrug resistance profiles were identified: PEN-FOX-TET-CTT-CIP-CHL-ERY-CLI-GEN-QDA and PEN-FOX-TET-CTT-CIP-CHL-ERY-CLI-GEN-QDA-SXT, which accounted for 39.5% (15/38) and 36.8% (14/38) of all MRSA isolates, respectively. The majority of MSSA isolates (134/162, 82.7%) were resistant to six or more antibiotics. Compared with MRSA isolates, the resistance profiles of MSSA isolates were more diverse. Twenty-six antibiotic profiles were identified and PEN-TET-CTT-CIP-ERY-CLI-GEN-CHL, PEN-TET-CTT-CIP-ERY-CLI-GEN and PEN-TET-CIP-ERY-CLI-GEN accounted for 59.9% (97/162) of all resistance profiles in MSSA isolates. All MRSA and seven MSSA isolates were resistant to quinupristin/dalfopristin.

Furthermore, PCR analysis revealed that 44 of 45 quinupristin/dalfopristin resistant strains were positive for the *lsa*(E) gene. In contrast, the remaining 1 isolate harbored none of the currently known quinupristin/dalfopristin resistance genes. ST9 was the only ST type among the 44 *lsa(E)* positive strains of which 7 (7/44,15.9%) were MSSA and 37 were MRSA(37/44, 84.1%)(Table S2). Two *spa* types were observed of which t899 was the dominant type among 100% (7/7) *lsa(E)* positive MSSA and 86.5% (32/37) *lsa(E)* positive MRSA strains and t2922 corresponded to 10.8% (4/37) *lsa(E)* positive MRSA strains.

Discussion

Heilongjiang is an important agricultural province in China. In 2011, 16 million pigs were raised for food production in this province alone. Until now, no data have been available on the occurrence of MRSA and MSSA in pigs from this area. The prevalence of MRSA (38/590, 6.4%) in pigs observed in this study was lower than the prevalence in other regions in China (Cui et al., 2009; Wang et al., 2012), Europe (Tenhagen et al., 2009; de Neeling et al., 2007; Gomez-Sanz et al., 2010) and North America (Smith et al., 2009; Khanna et al., 2008), but higher than the prevalence in some other Asian countries (Lim et al., 2012; Neela et al., 2009; Baba et al., 2010). Despite this moderate frequency of MRSA among slaughter pigs in our

Table 3. Antimicrobial susceptibility of Methicillin-resistant *Staphylococcus aureus* (MRSA) and Methicillin-resistant *S. aureus* (MSSA) from slaughter pigs

Antimicrobial agent	MRSA(38)				MSSA(162)			
	MIC range(mg/L)	MIC_{50}(mg/L)	MIC_{90}(mg/L)	%R	MIC range(mg/L)	MIC_{50}(mg/L)	MIC_{90}(mg/L)	%R
penicillin		64	128	100	0.064-8	2	4	99
cefoxitin	32-256	64	128	100	2-4	4	4	0
tetracycline	16-128	64	64	100	0.125-128	64	128	92
chlortetracycline	32-64	32	64	100	0.5-64	32	64	70
ciprofloxacin	1-64	16	32	97	0.5-64	32	>32	88
chloramphenicol	8-64	64	64	97	4-128	8	64	45
erythromycin	512	>256	>256	100	0.25-512	>256	>256	89
clindamycin	512	>256	>256	100	0.0625-512	>256	>256	88
gentamicin	16-64	32	64	100	0.125-128	16	64	79
rifampicin	0.002-8	0.016	8	16	0.002-4	0.004	0.06	1

trimethoprim/sulfamethoxazole	0.06-64	2	8	37	0.016-64	0.125	4	13
nitrofurantoin	16-512	32	>256	21	8-32	32	32	0
quinupristin/dalfopristin	4-8	4	8	100	0.25-4	0.5	2	4
vancomycin	0.5-2	1	1	0	0.5-2	1	1	0
teicoplanin	0.25-1	1	1	0	0.5-4	1	1	0
linezolid	1-4	2	2	0	0.75-4	2	4	0
tigecycline	0.38-0.5	0.5	0.5	0	0.094-0.5	0.19	0.38	0
daptomycin	0.19-0.5	0.25	0.38	0	0.094-0.38	0.125	0.19	0
fusidic acid	0.25-0.5	0.25	0.5	0	0.125-0.25	0.25	0.25	0
fosfomycin	4-16	8	16	0	1-16	4	8	0
mupirocin	0.125-2	0.125	0.25	0	0.125-38	0.25	0.5	0

study, multiple drug resistance (MDR) phenotype defined as resistance against 6 or more antibiotic was 86% (172/200) among all isolates suggestive of a significant reservoir of antibiotic resistance among farm animals in China.

Differences were observed in the prevalence of MRSA and the number of farms with MRSA between the two slaughterhouses. It can be speculated that the origins of these pigs and their rearing patterns were different. Slaughterhouse 1 was much bigger than slaughterhouse 2. Most of the pigs in slaughterhouse 1 came from larger farms, while in slaughterhouse 2 most of the pigs came from smaller farms where animals were kept free-range. Although MRSA carriage and transmission among pigs may not be a significant problem in this region, monitoring the occurrence of resistance in pigs may be advisable because certain clones may have the propensity to expand in the human population.

This is the first study to report the genetic population structure of *S. aureus* (MRSA and MSSA) from slaughterhouse pigs in northeast China. We found that ST398 was the most frequent ST type (99/162, 61.1%) among MSSA, whereas MRSA with this sequence type, typically associated with pigs in Europe, was not identified. ST398 MSSA is also a frequent community and hospital-acquired infections associated strain among humans in China (Zhao et al., 2012; Wu et al., 2010; Chen et al., 2010). In Europe, ST398 MRSA is associated with animal origin and is occasionally transmitted to humans via occupational exposure to farm animals. Whole-genome sequencing approaches and microarrays analysis of a large amount of ST398 isolates from different host species suggested that ST398 was most likely of human origin, and was occasionally transmitted to livestock companied with acquisition of SCC*mec* (Price LB et al, 2012; Uhlemann et al, 2012). Our study did not sample persons in direct contact with pigs, such as slaughterhouse workers and farmers. Whether transmission between humans and pigs is occurring in China is still unknown, but such transmission is likely.

Resistance to multiple antibiotics, not only among MRSA isolates, but notably also among MSSA isolates, is a matter of concern. It suggests that a substantial amount of antimicrobials are used in pig farming for growth promotion, prophylactic or therapeutic purposes. A high prevalence of multiple antimicrobial resistance in pig isolates was also found in another province (Shanxi) (Wang et al., 2012), and an abundance of diverse antibiotic resistance genes (ARGs) have been found on Chinese pig farms (Zhu et al., 2013); in contrast, most MSSA in Chinese hospitals have maintained a high degree of susceptibility to most antimicrobial agents, mainly exhibiting erythromycin and clindamycin resistance (Xiao et al., 2011). No doubt, there remains a potential threat to human health that resistant strains or

resistance genes that have emerged in pigs (Zhu et al., 2013) are introduced into human health care institutions.

In this study, all quinupristin/dalfopristin resistant strains belonged to ST9. This may be the result of clonal expansion most likely originating from breeding farms and the selection of ST9 by the use of virginiamycin against which the quinupristin/dalfopristin resistant isolates exhibit cross-resistance or deficiency of DNA restriction systems likely to facilitate the horizontal gene transfer of "foreign" DNA, as has been found in ST398 (Schijffelen et al., 2010). Virginiamycin belongs to the same antibiotic group (streptogramin) as quinupristin/dalfopristin. This compound has been widely used for more than 25 years as an animal growth promoter (AGP) in poultry, cattle and swine. Because of concerns that cross resistance between virginiamycin and quinupristin/dalfopristin could lead to treatment failure in humans, virginiamycin was banned from animal use in Europe. In China, however, it is still widely used. In our study, 98% (44/45) of the quinupristin/dalfopristin resistant strains harbored the new resistance gene, *lsa(E)*, which was first described recently (Wendlandt et al., 2013; Li et al., 2013). High homology between the sequence of the new transposon carrying *lsa(E)* in *S. aureus* and the sequence of the *Enterococcus faecalis* plasmid pEF418 demostrated the horizontal gene transfer interspecies (Wendlandt et al., 2013). So far, the gene *lsa(E)* has been only described in ST9 strains (both MRSA and MSSA) in China, but different *spa* types, antibiotic resistance profile and genomic location of the gene *lsa(E)* were observed. Whether the transposon has entered ST9 only once and then diversified and spread to different regions in China or, more likely, that the transposon prefers the ST9 background and has been aquired by ST9 on several occasions still need to be explained.

To our surprise, ST398 MSSA was the predominant clone among slaughterhouse pigs in northeast China. We hope that this finding will improve the understanding of the global population history of ST398.

Acknowledgements

This work was supported by the Major Infectious Diseases Such as AIDS and Viral Hepatitis Prevention and Control Technology Major Projects (2008ZX10004-002 and 2008ZX10004-008) of the Ministry of Science and Technology, People's Republic of China, by the State Key Laboratory for Infectious Disease Prevention and Control Fund (2012SKLID202), and by an educational grant from the Graduate School of Medical Sciences of the University of Groningen, the Netherlands.

References list

Armand-Lefevre, L., Ruimy, R., Andremont, A., 2005. Clonal comparison of *Staphylococcus aureus* isolates from healthy pig farmers, human controls, and pigs. Emerg. Infect. Dis. 11, 711-714.
Baba, K., Ishihara, K., Ozawa, M., Tamura, Y., Asai, T., 2010. Isolation of meticillin-resistant *Staphylococcus aureus* (MRSA) from swine in Japan. Int. J. Antimicrob. Agents. 36, 352-354.
Bens, C.C., Voss, A., Klaassen, C.H., 2006. Presence of a novel DNA methylation enzyme in methicillin-resistant *Staphylococcus aureus* isolates associated with pig farming leads to uninterpretable results in standard pulsed-field gel electrophoresis analysis. J. Clin. Microbiol. 44, 1875-1876.
Brakstad, O.G., Aasbakk, K., Maeland, J.A., 1992. Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the *nuc* gene. J. Clin. Microbiol. 30, 1654-1660.
Chen, H., Liu, Y., Jiang, X., Chen, M., Wang, H., 2010. Rapid change of methicillin-resistant *Staphylococcus aureus* clones in a Chinese tertiary care hospital over a 15-year period. Antimicrob. Agents Chemother. 54, 1842-1847.
CLSI., 2010. *Performance Standards for Antimicrobial Susceptibility Testing; Twentieth Informational Supplement.* CLSI document M100-S20. Wayne, PA: Clinical and Laboratory Standards Institute, pp.60-68
Cui, S., Li, J., Hu, C., Jin, S., Li, F., Guo, Y., Ran, L., Ma, Y., 2009. Isolation and characterization of methicillin-resistant *Staphylococcus aureus* from swine and workers in China. J. Antimicrob. Chemother. 64, 680-683.
de Neeling, A.J., van den Broek, M.J., Spalburg, E.C., van Santen-Verheuvel, M.G., Dam-Deisz, W.D., Boshuizen, H.C., van de Giessen, A.W., van, D.E., Huijsdens, X.W., 2007. High prevalence of methicillin resistant *Staphylococcus aureus* in pigs. Vet. Microbiol. 122, 366-372.
Enright, M.C., Day, N.P., Davies, C.E., Peacock, S.J., Spratt, B.G., 2000. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. J. Clin. Microbiol. 38, 1008-1015.
Finlay, J.E., Miller, L.A., Poupard, J.A., 1997. Interpretive criteria for testing susceptibility of staphylococci to mupirocin. Antimicrob. Agents Chemother. 41, 1137-1139.
Garcia-Alvarez, L., Holden, M.T., Lindsay, H., Webb, C.R., Brown, D.F., Curran, M.D., Walpole, E., Brooks, K., Pickard, D.J., Teale, C., Parkhill, J., Bentley, S.D., Edwards, G.F., Girvan, E.K., Kearns, A.M., Pichon, B., Hill, R.L., Larsen, A.R., Skov, R.L., Peacock, S.J., Maskell, D.J., Holmes, M.A., 2011. Meticillin-resistant *Staphylococcus aureus* with a novel *mecA* homologue in human and bovine populations in the UK and Denmark: a descriptive study. Lancet Infect. Dis. 11, 595-603.
Gomez-Sanz, E., Torres, C., Lozano, C., Fernandez-Perez, R., Aspiroz, C., Ruiz-Larrea, F., Zarazaga, M., 2010. Detection, molecular characterization, and clonal diversity of methicillin-resistant *Staphylococcus aureus* CC398 and CC97 in Spanish slaughter pigs of different age groups. Foodborne. Pathog. Dis. 7, 1269-1277.
Harmsen, D., Claus, H., Witte, W., Rothganger, J., Claus, H., Turnwald, D., Vogel, U., 2003. Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for *spa* repeat determination and database management. J. Clin. Microbiol. 41, 5442-5448.
Holden, M.T., Hsu, L.Y., Kurt, K., Weinert, L.A., Mather, A.E., Harris, S.R., Strommenger, B., Layer, F., Witte, W., de, L.H., Skov, R., Westh, H., Zemlickova, H., Coombs, G., Kearns, A.M., Hill, R.L., Edgeworth, J., Gould,I., Gant, V., Cooke, J., Edwards, G.F., McAdam, P.R., Templeton, K.E., McCann, A., Zhou, Z., Castillo-Ramirez, S., Feil, E.J., Hudson, L.O., Enright, M.C., Balloux, F., Aanensen, D.M., Spratt, B.G., Fitzgerald, J.R., Parkhill, J., Achtman, M., Bentley, S.D., Nubel, U., 2013. A genomic portrait of the emergence, evolution, and global spread of a methicillin-resistant *Staphylococcus aureus* pandemic. Genome Res. 23,

653-664.
Khanna, T., Friendship, R., Dewey, C., Weese, J.S., 2008. Methicillin resistant *Staphylococcus aureus* colonization in pigs and pig farmers. Vet. Microbiol. 128, 298-303.
Li, B., Wendlandt, S., Yao, J., Liu, Y., Zhang, Q., Shi, Z., Wei, J., Shao, D., Schwarz, S., Wang, S., Ma, Z., 2013. Detection and new genetic environment of the pleuromutilin-lincosamide-streptogramin A resistance gene *lsa(E)* in methicillin-resistant *Staphylococcus aureus* of swine origin. J. Antimicrob. Chemother. 68, 1251-1255.
Lim, S.K., Nam, H.M., Jang, G.C., Lee, H.S., Jung, S.C., Kwak, H.S., 2012. The first detection of methicillin-resistant *Staphylococcus aureus* ST398 in pigs in Korea. Vet. Microbiol. 155, 88-92.
Lina, G., Piemont, Y., Godail-Gamot, F., Bes, M., Peter, M.O., Gauduchon, V., Vandenesch, F., Etienne, J., 1999. Involvement of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. Clin. Infect. Dis. 29, 1128-1132.
MacGowan, A.P., Wise, R., 2001. Establishing MIC breakpoints and the interpretation of in vitro susceptibility tests. J. Antimicrob. Chemother. 48 Suppl 1, 17-28.
Mellmann, A., Weniger, T., Berssenbrugge, C., Rothganger, J., Sammeth, M., Stoye, J., Harmsen, D., 2007. Based Upon Repeat Pattern (BURP): an algorithm to characterize the long-term evolution of *Staphylococcus aureus* populations based on *spa* polymorphisms. BMC. Microbiol. 7, 98.
Morgan, M., 2008. Methicillin-resistant *Staphylococcus aureus* and animals: zoonosis or humanosis? J. Antimicrob. Chemother. 62, 1181-1187.
Neela, V., Mohd, Z.A., Mariana, N.S., van, B.A., Liew, Y.K., Rad, E.G., 2009. Prevalence of ST9 methicillin-resistant *Staphylococcus aureus* among pigs and pig handlers in Malaysia. J. Clin. Microbiol. 47, 4138-4140.
Price, L.B., Stegger, M., Hasman, H., Aziz, M., Larsen, J., Andersen, P.S., Pearson, T., Waters, A.E., Foster, J.T., Schupp, J., Gillece, J., Driebe, E., Liu, C.M., Springer, B., Zdovc, I., Battisti, A., Franco, A., Zmudzki, J., Schwarz, S., Butaye, P., Jouy, E., Pomba. C., Porrero, M.C., Ruimy, R., Smith, T.C., Robinson, D.A, Weese, J.S., Arriola, C.S., Yu, F., Laurent, F., Keim, P, Skov, R, Aarestrup, F.M., 2012.*Staphylococcus aureus* CC398: host adaptation and emergence of methicillin resistance in livestock. MBio. 3, e00305-e00311.
Schijffelen, M.J., Boel, C.H., van Strijp, J.A., Fluit, A.C., 2010. Whole genome analysis of a livestock-associated methicillin-resistant *Staphylococcus aureus* ST398 isolate from a case of human endocarditis. BMC. Genomics. 11, 376.
Sergio, D.M., Koh, T.H., Hsu, L.Y., Ogden, B.E., Goh, A.L., Chow, P.K., 2007. Investigation of meticillin-resistant *Staphylococcus aureus* in pigs used for research. J. Med. Microbiol. 56, 1107-1109.
Smith, T.C., Male, M.J., Harper, A.L., Kroeger, J.S., Tinkler, G.P., Moritz, E.D., Capuano, A.W., Herwaldt, L.A., Diekema, D.J., 2009. Methicillin-resistant *Staphylococcus aureus* (MRSA) strain ST398 is present in midwestern U.S. swine and swine workers. PLoS. One. 4, e4258.
Tenhagen, B.A., Fetsch, A., Stuhrenberg, B., Schleuter, G., Guerra, B., Hammerl, J.A., Hertwig, S., Kowall, J., Kampe, U., Schroeter, A., Braunig, J., Kasbohrer, A., Appel, B., 2009. Prevalence of MRSA types in slaughter pigs in different German abattoirs. Vet. Rec. 165, 589-593.
Uhlemann, A.C., Porcella, S.F., Trivedi, S., Sullivan, S.B., Hafer, C., Kennedy, A.D., Barbian, K.D., McCarthy, A.J., Street, C., Hirschberg, D.L., Lipkin, W.I., Lindsay, J.A., DeLeo, F.R., Lowy, F.D., 2012. Identification of a highly transmissible animal-independent *Staphylococcus aureus* ST398 clone with distinct genomic and cell adhesion properties. MBio. 3, e00012-e00027.
Van Cleef, B.A., Monnet, D.L., Voss, A., Krziwanek, K., Allerberger, F., Struelens, M., Zemlickova, H., Skov, R.L., Vuopio-Varkila, J., Cuny, C., Friedrich, A.W., Spiliopoulou, I., Paszti, J., Hardardottir, H., Rossney, A., Pan, A., Pantosti, A., Borg, M., Grundmann, H., Mueller-Premru, M., Olsson-Liljequist, B., Widmer, A., Harbarth, S., Schweiger, A., Unal, S., Kluytmans, J.A., 2011. Livestock-associated methicillin-resistant *Staphylococcus aureus* in

humans, Europe. Emerg. Infect. Dis. 17, 502-505.
Vanderhaeghen, W., Hermans, K., Haesebrouck, F., Butaye, P., 2010. Methicillin-resistant *Staphylococcus aureus* (MRSA) in food production animals. Epidemiol. Infect. 138, 606-625.
Voss, A., Loeffen, F., Bakker, J., Klaassen, C., Wulf, M., 2005. Methicillin-resistant *Staphylococcus aureus* in pig farming. Emerg. Infect. Dis. 11, 1965-1966.
Wagenaar, J.A., Yue, H., Pritchard, J., Broekhuizen-Stins, M., Huijsdens, X., Mevius, D.J., Bosch, T., Van, D.E., 2009. Unexpected sequence types in livestock associated methicillin-resistant *Staphylococcus aureus* (MRSA): MRSA ST9 and a single locus variant of ST9 in pig farming in China. Vet. Microbiol. 139, 405-409.
Wang, X., Meng, J., Zhou, T., Zhang, Y., Yang, B., Xi, M., Sheng, J., Zhi, S., Xia, X., 2012. Antimicrobial susceptibility testing and genotypic characterization of *Staphylococcus aureus* from food and food animals. Foodborne. Pathog. Dis. 9, 95-101.
Wendlandt, S., Lozano, C., Kadlec, K., Gomez-Sanz, E., Zarazaga, M., Torres, C., Schwarz, S., 2013. The enterococcal ABC transporter gene *lsa(E)* confers combined resistance to lincosamides, pleuromutilins and streptogramin A antibiotics in methicillin-susceptible and methicillin-resistant *Staphylococcus aureus.* J. Antimicrob. Chemother. 68, 473-475.
Witte, W., Strommenger, B., Stanek, C., Cuny, C., 2007. Methicillin-resistant *Staphylococcus aureus* ST398 in humans and animals, Central Europe. Emerg. Infect. Dis. 13, 255-258.
Wu, D., Wang, Q., Yang, Y., Geng, W., Wang, Q., Yu, S., Yao, K., Yuan, L., Shen, X., 2010. Epidemiology and molecular characteristics of community-associated methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* from skin/soft tissue infections in a children's hospital in Beijing, China. Diagn. Microbiol. Infect. Dis. 67, 1-8.
Xiao, Y.H., Giske, C.G., Wei, Z.Q., Shen, P., Heddini, A., Li, L.J., 2011. Epidemiology and characteristics of antimicrobial resistance in China. Drug Resist. Updat. 14, 236-250.
Zhang, K., McClure, J.A., Elsayed, S., Louie, T., Conly, J.M., 2005. Novel multiplex PCR assay for characterization and concomitant subtyping of staphylococcal cassette chromosome *mec* types I to V in methicillin-resistant *Staphylococcus aureus*. J. Clin. Microbiol. 43, 5026-5033.
Zhang, W., Hao, Z., Wang, Y., Cao, X., Logue, C.M., Wang, B., Yang, J., Shen, J., Wu, C., 2011. Molecular characterization of methicillin-resistant *Staphylococcus aureus* strains from pet animals and veterinary staff in China. Vet. J. 190, e125-e129.
Zhao, C., Liu, Y., Zhao, M., Liu, Y., Yu, Y., Chen, H., Sun, Q., Chen, H., Jiang, W., Liu, Y., Han, S., Xu, Y., Chen, M., Cao, B., Wang, H., 2012. Characterization of community acquired *Staphylococcus aureus* associated with skin and soft tissue infection in Beijing: high prevalence of PVL^+ ST398. PLoS. One. 7, e38577.
Zhu, Y.G., Johnson, T.A., Su, J.Q., Qiao, M., Guo, G.X., Stedtfeld, R.D., Hashsham, S.A., Tiedje, J.M., 2013. Diverse and abundant antibiotic resistance genes in Chinese swine farms. Proc. Natl. Acad. Sci. U. S. A. 110, 3435-3440.

Supplementary Materials

Table S1. Primer sequences used for detection of quinupristin/dalfopristin resistance genes

Primer	Sequence (5'→3')	Reference
vat(A)-F	AGAATTAGAAGTACGTCTAAATGC	(Soltani et al., 2000)
vat(A)-R	GCTGATTATGTAAATGACTCAAATG	
vat(B)-F	GGCCCTGATCCAAATAGCAT	(Soltani et al., 2000)
vat(B)-R	GTGCTGACCAATCCCACCAT	
vat(C)-F	ATGAATTCGCAAAATCAGCAAGG	(Soltani et al., 2000)
vat(C)-R	TCGTCTCGAGCTCTAGGTCC	
vga(A)-F	AGTGGTGGTGAAGTAACACG	(Soltani et al., 2000)
vga(A)-R	CTTGTCTCCTCCGCGAATAC	
vga(A)variant-F	CTCTTTGTACGAGTATATGG	(Haroche et al., 2003)
vga(A)variant-R	GTTTCTTAGTAGCTCGTTGAGC	
vga(B)-F	TGACAATATGAGTGGTGGTG	(Soltani et al., 2000)
vga(B)-R	GCGACCATGAAATTGCTCTC	
vga(C)-F	AGCTGGAGAAAGCAATCCAA	(Argudin et al., 2011)
vga(C)-R	TCTTGCTGAAAATCCCGTTC	
vga(E)-F	GAAATATGGGAAATAGAAGATGG	(Schwendener and Perreten, 2011)
vga(E)-R	TGATTCTCTAACCACTCTTC	
cfr-F	TGAAGTATAAAGCAGGTTGGGAGTCA	(Shore et al., 2010)
cfr-R	ACCATATAATTGACCACAAGCAGC	
lsa(E)-F	ACGGACGCGGTAAAACTACT	The present study
lsa(E)-R	AGGACCTTCGTTTGCTCACC	

Table S2. Characteristics of quinupristin/dalfopristin resistant strains with the *lsa(E)* gene from slaughter pigs.

MSSA/MRSA	MLST	*spa* type	Antibiotic resistance
MSSA	ST9(7)	t899(7)	PEN-CHL-TET-GEN-CIP-ERY-CLI-CTT-QDA(2)
			PEN-CHL-TET-GEN-CIP-ERY-CLI-SXT-CTT-QDA(4)
			PEN-TET-GEN-CIP-ERY-CLI-SXT-CTT-QDA(1)
MRSA	ST9(37)	t899(32)	PEN-FOX-CHL-TET-GEN-CIP-ERY-CLI-CTT-QDA(9)
			PEN-FOX-CHL-TET-GEN-CIP-ERY-CLI-NIT-CTT-QDA(1)
			PEN-FOX-CHL-TET-GEN-CIP-ERY-CLI-NIT-RIF-CTT-QDA(1)
			PEN-FOX-CHL-TET-GEN-CIP-ERY-CLI-SXT-CTT-QDA(12)
			PEN-FOX-GEN-CIP-CLI-NIT-RIF-MUP-CTT-QDA(1)
			PEN-FOX-GEN-CLI-NIT-RIF-MUP-CTT-QDA(5)
			PEN-FOX-TET-GEN-CIP-CLI-NIT-RIF-MUP-CTT-QDA(1)
			PEN-FOX-TET-GEN-CIP-ERY-CLI-CTT-QDA(1)
			PEN-FOX-TET-GEN-CIP-ERY-CLI-SXT-CTT-QDA(1)
		t2922(4)	PEN-FOX-CHL-TET-GEN-CIP-ERY-CLI-CTT-QDA(2)
			PEN-FOX-CHL-TET-GEN-CIP-ERY-CLI-SXT-CTT-QDA(2)
		Non-typable(1)	PEN-FOX-TET-GEN-CIP-ERY-CLI-CTT-QDA(1)

PEN, penicillin; FOX, cefoxitin; TET, tetracycline; CTT, chlortetracycline; CIP, ciprofloxacin; CHL, chloramphenicol; ERY, erythromycin; CLI, clindamycin; GEN, gentamicin; RIF, rifampin; SXT, trimethoprim/sulfamethoxazole; NIT, nitrofurantoin; QDA, quinupristin/dalfopristin; MUP, mupirocin.

Reference list

Argudin,M.A., Tenhagen,B.A., Fetsch,A., Sachsenroder,J., Kasbohrer,A., Schroeter,A., Hammerl,J.A., Hertwig,S., Helmuth,R., Braunig,J., Mendoza,M.C., Appel,B., Rodicio,M.R., Guerra,B., 2011. Virulence and resistance determinants of German *Staphylococcus aureus* ST398 isolates from nonhuman sources. Appl. Environ. Microbiol. 77, 3052-3060.

Haroche,J., Morvan,A., Davi,M., Allignet,J., Bimet,F., El,S.N., 2003. Clonal diversity among streptogramin A-resistant *Staphylococcus aureus* isolates collected in French hospitals. J. Clin. Microbiol. 41, 586-591.

Schwendener,S., Perreten,V., 2011. New transposon Tn6133 in methicillin-resistant *Staphylococcus aureus ST398* contains *vga(E)*, a novel streptogramin A, pleuromutilin, and lincosamide resistance gene. Antimicrob. Agents Chemother. 55, 4900-4904.

Shore,A.C., Brennan,O.M., Ehricht,R., Monecke,S., Schwarz,S., Slickers,P., Coleman,D.C., 2010. Identification and characterization of the multidrug resistance gene *cfr* in a Panton-Valentine leukocidin-positive sequence type 8 methicillin-resistant *Staphylococcus aureus* IVa (USA300) isolate. Antimicrob. Agents Chemother. 54, 4978-4984.

Soltani,M., Beighton,D., Philpott-Howard,J., Woodford,N., 2000. Mechanisms of resistance to quinupristin-dalfopristin among isolates of *Enterococcus faecium* from animals, raw meat, and hospital patients in Western Europe. Antimicrob. Agents Chemother. 44, 433-436.

Chapter 5

Factors Associated with *Staphylococcus aureus* Nasal Carriage among Healthy People in Northern China

Xiaomei Yan[#], Yanyan Song[#], Xiaojie Yu[#], Xiaoxia Tao, Jun Yan, Fengji Luo, Huifang Zhang, Jianfeng Zhang, Qian Li, Lihua He, Shuming Li, Fanliang Meng, Jianzhong Zhang*, Hajo Grundmann*

[#] These authors contributed equally to this work

Published in the *Clin Microbiol Infect* (2015)21,157-62

Abstract

There is still limited knowledge about the prevalence and risk factors of nasal carriage for *Staphylococcus aureus* among healthy carriers in China. We investigated 2448 healthy adults (≥18 y) from Beijing (1530) and Harbin (918) by nasal screening. Participants were checked for carriage of *S. aureus*, and health-related and demographic information between 2009 and 2011 was gathered. A total of 403 *S. aureus* (403/2448, 16.5%) were recovered, of which 8 were methicillin-resistant *S. aureus* (MRSA) (8/2448, 0.33%). Three factors were independently associated with *S. aureus* nasal carriage, which included city of residence, Harbin (OR=2.0, 95% CI=1.41 to 2.85), age ≤ 24 years (OR=1.77, 95% CI=1.30-2.44) and non-Han ethnicity (OR=1.58, 95% CI=1.05 to 2.38). Based on population genetic analysis using multiple locus variable number of tandem repeats analysis (MLVA) and *spa*-typing, MLVA complex (MC) 398 and MC5a were the most prevalent clonal lineages in this collection. In multivariate models, residing in Harbin (OR=1.77, 95% CI=1.07-2.92) and having household members in the healthcare profession (OR=3.69, 95% CI=1.14-11.92) are factors associated with carriage of clonal lineage MC398. On the other hand, female sex (OR=3.15, 95% CI=1.35-7.33) and a history of chronic liver disease (OR=16.93, 95% CI=2.91-98.59) were associated with the clonal lineage MC5a. The three most common *spa* types were t571 (10.9%), t189 (9.9%) and t701 (7.2%). These findings provide insights into the determinants of nasal carriage and ecology for some of the most successful strains of *S. aureus* among healthy people in Northern China.

Introduction

Staphylococcus aureus is a leading cause of hospital-associated (HA) and community-onset (CO) bacterial infections in humans. The most important factors which contribute to the success of *S. aureus* as a pathogen are believed to be i) the ability to persist as a commensal, ii) resistance to multiple antimicrobial agents and iii) the diverse repertoire of virulence determinants [1,2].

S. aureus can colonise multiple sites of the human body, but the anterior nares appear to be the main ecological niche [3]. In healthy Caucasians it is estimated that 20% are persistent carriers and an additional 30% intermittent carriers, while approximately 50% are non-carriers. There are three lines of evidence that support the view that *S. aureus* nasal carriage is associated with a higher chance to develop staphylococcal infections. First, the rates of infection are higher in persistent carriers than others [4]. Second, high resolution molecular typing using pulsed-field gel electrophoresis (PFGE) has shown that infecting strains of *S. aureus* were indistinguishable from carriage isolates previously isolated from external nares of patients who later developed an invasive infection [5,6]. Finally, eradication of this microorganism is regarded as an effective means for reducing infections in surgical and dialysis patients [4, 7].

Over the past decade, reports about community-onset methicillin-resistant *S. aureus* (MRSA) infections have raised concern about the public health implications of *S. aureus* transmission among healthy individuals. Therefore, unravelling the risk factors for carriage of *S. aureus* is crucial for understanding the transmission potential of both MRSA and methicillin-sensitive *S. aureus* (MSSA). To date, there have been only few reports on the prevalence and the risk factors of *S. aureus* nasal carriage in China. Previous studies revealed 15.4-23.1% *S. aureus* nasal carriage in Chinese medical students from different regions, of which 3.0-9.4% were MRSA [8, 9]. Another study revealed a similar nasal carriage rate (20%) in 1,044 military volunteers from Beijing whereby no MRSA strains were identified [10]. Both studies focused on populations who typically live under crowded conditions and thereby had higher opportunities for transmission. It still remains unclear whether carriage rates and risk factors among the Chinese general population are in the same range. We therefore performed a population-based survey to determine the prevalence and risk factors of *S. aureus* nasal carriage in two cities from Northern China.

Materials and methods

Population and study design

A cross-sectional study was conducted in two northern cities, namely, Beijing and Harbin. Individuals presenting for mandatory occupational health screens from food and public service industries as well as public health workers were chosen as healthy volunteers for the present survey. Between 2009 and 2011, 1530 and 918 nasal swabs were sampled in Beijing and Harbin, respectively. A self-administered questionnaire was completed by each volunteer to collect pertinent demographic and medical information, and factors that are potentially related to *S. aureus* nasal carriage and transmission as identified by the literature.

Bacterial strains

Nasal swabs from both nares were enriched in Tryptic Soy Broth (OXOID, Basingstoke, England) with 7% NaCl at 37℃ for 24 h and then plated onto Mannitol Salt Agar (OXOID, Basingstoke, England), and culture at 37℃ for 24 h. Presumptive *S. aureus* colonies were confirmed by colony morphology, Gram staining, catalase production, coagulase production using the Slidex Staph Plus kit (Murex Biotech, Kent, France) and PCR for the *nuc* and *mecA* gene [11].

Molecular typing and Panton-Valentine leukocidin (PVL) gene detection

Multiple-locus variable-number of tandem repeats analysis (MLVA) was carried out for all 403 isolates and representative strains of each MLVA complex were analysed by multiple-locus sequence typing (MLST) in a previous study (not published). All the isolates were also characterised using *spa* typing [12]. The *pvl* gene was detected by PCR, as previously described [13].

Simpson's index of diversity and 95% confidence intervals were used to calculate the genetic diversity of strains by Ridom EpiCompare software version 1.0 (Ridom GmbH, Münster, Germany).

Potential Risk factors

Several variables were investigated as potential risk factors of *S. aureus* nasal carriage. These included general demographic variables such as age, sex, ethnicity, and immune system impairment. Immune system impairment was defined as diagnosis of primary and secondary immunodeficiency disorders, and systemic immune suppressive therapy was also included. Additional risk factors such as skin and soft-tissue infections, hospitalisation, use of antibiotics, and frequent contact with animals or animal products in the past 6 months, were also recorded. At the same time, we were also interested in transmission between family members at household level. Therefore, risk factors of other household members were also

investigated (Appendix S1).

Statistical analysis

The questionnaires were manually imported into EpiData (v3.02) software (EpiData Association, Odense, Denmark). Statistical comparisons were performed with SPSS (PASW Statistics 18.0.3) software. The only continuous variable, age, was transformed into a categorical variable using the quartiles of the frequency distribution (≤24, >24≤30, >30≤40, >40). Furthermore, the different occupations were grouped into four categories which involves: i) physical contact with healthy people (such as hairdressing and kindergarten teacher); ii) physical contact with animals or animal products (such as animal handlers, meat processing personnel, cooks, etc.); iii) physical contact with patients and patient material (such as healthcare personnel); and iv) nonphysical contact with people or animals (remaining occupations). Categorical variables were compared using the chi-square test or Fisher exact test. Odds ratios (OR), 95% confidence intervals (CI), and P-values were calculated. A P-value of ≤0.05 was considered statistically significant. Logistic regression models were applied to determine independent risk factors. Multiple logistic regression analysis was carried out by entering all the independent variables associated with the outcome with an alpha error accepted to the level of P<0.2.

Ethical considerations

The study was approved by the ethical committee of the National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention. Furthermore, written consent was obtained from all participants.

Results

Characteristics of study subjects

From 2009 to 2011, a total of 2448 volunteers from two cities were enrolled into this study, of which 1530 were from Beijing and 918 were from Harbin. The majority of the volunteers were female (1617/2446, 66.1%). Ages ranged from 18 to 74 years (mean, standard deviation: 32.4, 10.3). Of the 2448 volunteers, 403 (16.5%) carried *S. aureus,* including 8 MRSA carriers (0.33%). *S. aureus* carriage in Harbin (197/918, 21.46%) was more frequent than in Beijing (206/1530, 13.46%) (P<0.001).

Epidemiological factors associated with *S. aureus* carriage

Variables associated with *S. aureus* carriage in the univariate analysis are shown in Table 1. Statistically significant factors included: age ≤24 years; ethnicity, non-Han; residing in Harbin;

immune system impairment; and hospitalisation of a household member within the past year. Three independent risk factors remained associated in the multiple logistic regression analysis (Table 1). These consisted of city of residence, Harbin (OR=2.0, 95% CI=1.41 to 2.85), age ≤24 years (OR=1.77, 95% CI=1.30-2.44) and non-Han ethnicity (OR=1.58, 95% CI=1.05 to 2.38).

Factors associated with strains belonging to successful clonal lineages

In the present collection of MLVA complexes (MC), 398 and 5a were the most prevalent complexes representing 21.0% and 11.7% of all isolates. The present study tried to assess the factors associated with nasal carriage for strains belonging to these two lineages. Multivariate logistic regression showed that residing in Harbin (OR=1.77, 95% CI=1.07-2.92) and having household members in the healthcare profession (OR=3.69, 95% CI=1.14-11.92) were factors associated with increased risk of carrying MC398 strains (Table 2). On the other hand, being of female sex (OR=3.15, 95% CI=1.35-7.33) and having a history of chronic liver disease (OR=16.93, 95% CI=2.91-98.59) heightened risk associated with MC5a carriage (Table 3).

***Spa* typing and panton-valentine leukocidin *(pvl)* gene detection**

In total, the 403 isolates were assigned to *77 spa* types, including 10 novel *spa* types, where 56 *spa* types were identified in Beijing and 42 in Harbin. Nineteen *spa* types were found in both cities, corresponding to 69.98% of all isolates. The top 11 *spa* types in both cities are shown in Table 4. The most commonly encountered *spa* types in this study were t571/CC398/ST398/MC398 (10.9%), t189/CC1/ST2139/MC437 (9.9%) and t701/ST6/ST2114/MC1933 (7.2%). Furthermore, our analyses revealed that carriage isolates had the same genetic diversity in both sampling locations with a diversity index of 0.952 (0.941-0.964).

Moreover, the 8 MRSA strains belonged to different *spa* types. Two isolates had *spa* type t034/CC398/ST398/MC398, and one isolate each of *spa* type t2431/Singleton/ST2799/MC482, t437/CC59/ST59/MC621 and t116/CC45/MLST45/MC45. The remaining two MRSA isolates were not typeable by *spa* typing.

Noticeably, the *pvl* gene was only detected among nine MSSA isolates (9/403, 2.2%), of which two had *spa* type t002/CC5/ST5/MC5a, and one each of *spa* type

TABLE 1. Univariate and multivariate analysis of risk factors associated with *S. aureus* nasal carriage in 2446 healthy people in northern China during 2009-2011

Characteristic	No. (%) of carrier (n=400)	No. (%) of noncarrier (n=2046)	Univariate			Multivariate logistic		
			OR	95%CI	P value	OR	95%CI	P value
Sex, female (n=1617)	249 (15.4%)	1368 (84.6%)	0.82	0.65-1.02	0.073			
Age, years								
≤24 (n=661)	135 (20.4%)	526 (79.6%)	1.52	1.13-2.06	0.001	1.77	1.30-2.44	<0.001
>24≤30 (n=642)	101 (15.7%)	541 (84.3%)	1.11	0.81-1.52	0.526	1.30	0.94-1.80	0.116
>30≤40 (n=584)	84 (14.4%)	500 (85.6%)	1.00	0.72-1.39	0.988	1.01	0.72-1.43	0.939
Race, non-Han (n=147)	34 (23.1%)	113 (76.9%)	1.59	1.07-2.37	0.03	1.58	1.05-2.38	0.027
City of residence								
Beijing (n=1229)	171 (13.9%)	1058 (86.1%)	1.03	0.74-1.44	0.84	1.16	0.85-1.64	0.390
Harbin (n=820)	175 (21.3%)	645 (78.7%)	1.74	1.24-2.42	0.00	2.00	1.41-2.85	<0.001
Occupation								
Contact with animal product (n=775)	139 (17.9%)	636 (82.1%)	1.75	0.73-4.17	0.21			
Contact with patient people (n=66)	10 (15.2%)	56 (84.8%)	1.43	0.48-4.22	0.519			
Nonphysical contact with healthy people (n=1531)	240 (15.7%)	1291 (84.3%)	1.49	0.63-3.51	0.366			
Education								
Primary school (n=125)	16 (12.8%)	109 (87.2%)	0.34	0.08-1.46	0.148			
Middle school (n=1107)	184 (16.6%)	923 (83.4%)	0.47	0.12-1.82	0.271			
High school (n=791)	132 (16.7%)	659 (83.3%)	0.47	0.12-1.83	0.275			

University or higher (n=407)	64 (15.7%)	343 (84.3%)	0.44	0.11-1.73	0.237
Immunity system disease, yes (n=6)	3 (50%)	3 (50%)	5.16	1.04-25.65	0.045
Hospitalisation in past half year, yes (n=31)	7 (22.6%)	24 (77.4%)	1.51	0.65-3.53	0.343
Antibiotic use in past half year, yes (n=380)	66 (17.4%)	314 (82.6%)	1.09	0.82-1.46	0.561
Frequent skin puncture in past half a year, yes (n=126)	20 (15.9%)	106 (84.1%)	0.97	0.59-1.58	0.898
Regular contact sports activities, yes (n=229)	39 (17.0%)	190 (83.0%)	1.06	0.74-1.53	0.741
Smoking					
Ex-smokers (n=289)	41 (14.2%)	248 (85.8%)	0.83	0.58-1.18	0.295
Smokers (n=413)	67 (16.2%)	346 (83.8%)	0.97	0.73-1.30	0.835
Regular contact with living animals, yes (n=215)	32 (14.9%)	183 (85.1%)	0.89	0.60-1.31	0.548
Household member hospitalisation within 1 year, yes (n=97)	23 (23.7%)	74 (76.3%)	1.63	1.01-2.64	0.045

TABLE 2. Univariate and multivariate analysis of risk factors associated with the MC398 strain in 391 *S. aureus* carriers in northern China during 2009-2011

Characteristic	MC398 carrier (n=80)	Non-MC398 carrier (n=311)	Univariate			Multivariate logistic		
			OR	95%CI	P value	OR	95%CI	P value
Inhabitant city								
Harbin (n=168)	44 (26.2%)	124 (73.8%)	1.88	1.10-3.21	0.021	1.77	1.07-2.92	0.027
Other cities (n=52)	9 (17.3%)	43 (82.7%)	1.11	0.48-2.54	0.807			
Birth place								
Harbin (n=75)	22 (29.3%)	53 (70.7%)	3.53	1.12-11.13	0.032			
Other cities (n=278)	54 (19.4%)	224 (80.6%)	2.05	0.70-6.02	0.192			
Skin and soft-tissue infection in past half a year, yes (n=6)	3 (50%)	3 (50%)	3.97	0.79-20.08	0.095			
Hospitalisation in past half year, yes (n=7)	3 (42.9%)	4 (57.1%)	2.97	0.65-13.55	0.160			
Regular contact sports activities, yes (n=39)	12 (30.8%)	27 (69.2%)	1.84	0.89-3.81	0.103			
Household members who have a profession in healthcare, yes (n=13)	6 (46.2%)	7 (53.8%)	3.49	1.14-10.68	0.029	3.69	1.14-11.92	0.029

TABLE 3. Univariate and multivariate analysis of risk factors associated with the MC5a strain in 391 *S. aureus* carriers in northern China during 2009-2011

	MC5a carrier	Non-MC5a	Univariate			Multivariate logistic		
			OR	95%CI	P value	OR	95%CI	P value
Sex, female (n=241)	33 (13.7%)	208 (86.3%)	2.49	1.15-5.35	0.020	3.15	1.35-7.33	0.008
Race, non-Han (n=33)	6 (18.2%)	27 (81.8%)	1.99	0.77-5.14	0.156			
Chronic liver disease history, yes (n=6)	3 (50.0%)	3 (50%)	8.85	1.73-45.34	0.009	16.93	2.91-98.59	0.002
Smoking								
Non-smokers (n=285)	33 (11.6%)	252 (88.4%)	2.71	0.80-9.11	0.108			
Ex-smokers (n=40)	6 (15.0%)	34 (85.0%)	3.65	0.86-15.51	0.080			

TABLE 4. The top 11 *spa* types of *S. aureus* in two cities

Beijing				Harbin				Total		
spa type	Frequency	%	MLVA complex	*spa* type	Frequency	%	MLVA complex (No.)	*spa* type	Frequency	%
t189	24	11.65%	MC437 (18),	t571	27	13.71%	MC398 (27)	t571	44	10.92%
t701	20	9.71%	MC1933 (14),	t189	16	8.12%	MC437 (12), NM (4)	t189	40	9.93%
t002	18	8.74%	MC5a (17),	t034	14	7.11%	MC398 (12), MC674	t701	29	7.20%
t571	17	8.25%	MC398 (16),	t127	11	5.58%	MC1 (8), NM (3)	t002	27	6.70%
t796	10	4.85%	MC7 (9), NM	t002	9	4.57%	MC5a (9)	t034	23	5.71%
t437	10	4.85%	MC621 (10)	t701	9	4.57%	MC1933 (6), NM (2)	t127	21	5.21%
t127	10	4.85%	MC1 (8), NM	t078	8	4.06%	MC674 (7), NM (1)	t796	17	4.22%
t034	9	4.37%	MC398 (8),	t377	8	4.06%	MC8 (5), NM (3)	t437	16	3.97%
t091	8	3.88%	MC7 (8)	t796	7	3.55%	MC7 (5), NM (2)	t091	11	2.73%
t163	5	2.43%	MC621 (5)	t084	6	3.05%	MC15 (6)	t078	10	2.48%
t803	4	1.94%	MC15 (2), NM	t437	6	3.05%	MC621 (4), NM (2)	t084	8	1.99%
Total	135	65.53%		Total	121	61.42%		Total	246	61.04%

[a] NM, not belong to any MCs

t011/CC398/ST398/MC398, t091/CC7/ST7/MC7, t1376/CC88/ST2148/MC5b, t701/CC6/ST2114/MC1933, t167/CC5/ST25/MC674, t645/CC121/ST123/MC123, and t7611/CC22/ST22/MC22.

Discussion

We found nasal carriage with *S. aureus* in 16.5% of our study population. This finding coincides with the prevalence observed among recruits in a military camp (16%) [10] and medical college students in another study (15.4%) [9]. Cross-sectional studies conducted outside of China found prevalence estimates ranging from 8% to 37% among different populations, where 0-8.6% was MRSA [14, 15, 16, 17]. The low prevalence of MRSA (0.36%) and the heterogeneity of *spa* types suggest that there were no singularly expanding MRSA clones among the study population.

Higher *S. aureus* carriage rates were published for Caucasians [18,19], men [20], individuals with obesity [14], children [9] and people with underlying diseases [4], especially skin disorders. We identified a significantly higher carriage among adults who are ethnically non-Han Chinese. Previous studies have shown that the HLA-DR3 antigen predisposes healthy individuals and transplant patients to *S. aureus* nasal carriage with *S. aureus* [21, 22]. Thus, population-specific frequencies of human leukocyte antigen (HLA) haplotypes may explain differential susceptibilities between ethnic groups. However, HLA-DR3 haplotype frequencies among the non-Han *S. aureus* carriers have not been investigated.

Interestingly, our study showed that the presence of household members who worked in the healthcare sector were more at risk for carrying strains belonging to the MC398 lineage. As previous studies have shown, high concordance between *S. aureus* strains isolated from medical staff and inpatients [23]. Additionally, it has been found that up to 65% of *S. aureus* carriers living within one household share genotypically identical strains [3]. These findings may indicate that healthcare personnel could contribute to the dissemination of MC398 strains between hospitals and community, which should be paid more attention to.

The bacterial population of *S. aureus* carriage isolates in our sample collection demonstrated the dominance of two clonal lineages against a background of a large genetic diversity. This diversity is consistent with previous findings among clinical MSSA strains [24]. Not surprisingly, there was a good correlation between *spa* types in healthy people of this study and the *S. aureus* strains isolated from hospital patients and outpatients with SSTI infections [25]. Five of the top seven *spa* types also represented the most frequent *spa* types among

clinical isolates from patients with CA-MSSA and HA-MSSA infections [26]. This finding lends further support to the notion that *S. aureus* carriers are at risk of autoinfection.

In conclusion, our study showed that younger people (≤24 years) and ethnically non-Han individuals were more likely to be colonised by *S. aureus.* Furthermore, the presence of household members who are healthcare personnel appeared to be a risk factor for MC398 carriage. Importantly, approximately one third of all isolates showed the same *spa* types with CA-MSSA and HA-MSSA in some hospitals. These findings could be helpful for understanding the determinants of *S. aureus* nasal carriage and transmission routes of some successful strains in Northern China.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant No. 81301463), by the State Key Laboratory for Infectious Disease Prevention and Control Fund of China (2012SKLID202), and by an educational grant from the Graduate School of Medical Sciences of the University of Groningen, the Netherlands.

Supplementary Materials

Questionnaire

Questionnaire

Code：_____

Demographics

1. Name：
2. Sex: male＝1，female＝2； ☐
3. Folk：han＝1，non-han＝2 ☐
4. Birth date：_____ /_____/ ____（yyyy/mm/dd）
5. Resident： Permanent residence in the city＝1； non-Permanent residence in the city＝2 ☐
6. Address： Province city district (couty) street (village) number
7. Occupation: waiter/waitress =1；shoping salesman (store and supermarket)=2；kindergarten teachers =3；housekeeping =4；kitchener＝5；hairdressing=6； hotel service=7，Pharmacy sales =8，health care personnel =9, others=10 ☐
8. Education： illiteracy＝1；grade school＝2；junior high school＝3；senior high school＝4；above junior college＝5 ☐
9. Telephone（at lease one）

（1）mobile phone _____

（2）home tel _____

（3）office tel _____

Disease history：

diabetes=1 ; chronic liver disease =2; tuberculosis=3 ; coronary heart disease=4 ; nervous system disease =5; cancer=6 ; immune system impairment =7；frequent skin and soft-tissue infection =8; others=9 ☐

Risk factor (yes＝1，no＝2)

1. Do you have a previous hospitalization in past half a year? ☐
2. Do you have a frequent skin puncture (acupuncture, insulin injection, dialysis patient)？ ☐
3. Do you have a skin disease such as dermatitis or skin ulcers now? ☐
4. Do you have a wound that doesn't heal now? ☐
5. Antibiotic use within the previous 6 months? ☐
6. Do you have regular (daily) contact with living animals? ☐

(Daily: every day or at least four times every week?)

If no, please go to question (7)

If yes, what animals:_____

Is this contact professional (such as farmer, slaughterhouse worker, pet shop keeper, live market worker, etc)? ☐

Is this contact non-professional (such as Domestic pets, etc.)? ☐

7. Do you have daily contact with animal products such as raw meat, skins, and hides? ☐

If no, please go to question (8)

If yes, which products:_____

8. Do you have a household member who is health care worker? ☐

9. Do you have a household member who was admitted to a hospital within 1 year? ☐

10. Do you have a household member that has regular contact with animals? ☐

If no, please go to question (11)

If yes, what animals:_____

Is this contact professional? ☐

Is this contact non professional? ☐

11. Do you have a household member that has daily contact with animal products? ☐

If no, please go to question (12)

If yes, which products:_____

12. Do you often have competitive close contact sports activities such as wrestling, football etc? ☐

13. Smoking habits: smokers＝1, ex-smokers=2, non-smokers=3 ☐

Reference List

1. Otto M. Basis of virulence in community-associated methicillin-resistant *Staphylococcus aureus*. Annu Rev Microbiol 2010; 64: 143-162.
2. Chambers HF, Deleo FR. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. Nat Rev Microbiol 2009; 7: 629-641.
3. Wertheim HF, Melles DC, Vos MC *et al.* The role of nasal carriage in *Staphylococcus aureus* infections. Lancet Infect Dis 2005; 5: 751-762.
4. Yu VL, Goetz A, Wagener M *et al. Staphylococcus aureus* nasal carriage and infection in patients on hemodialysis. Efficacy of antibiotic prophylaxis. N Engl J Med 1986, 315: 91-96.
5. Wertheim HF, Vos MC, Ott A *et al.* Risk and outcome of nosocomial *Staphylococcus aureus* bacteraemia in nasal carriers versus non-carriers. Lancet 2004; 364: 703-705.
6. von EC, Becker K, Machka K, Stammer H, Peters G. Nasal carriage as a source of *Staphylococcus aureus* bacteremia. Study Group. N Engl J Med 2001; 344: 11-16.
7. Konvalinka A, Errett L, Fong IW. Impact of treating *Staphylococcus aureus* nasal carriers on wound infections in cardiac surgery. J Hosp Infect 2006; 64: 162-168.
8. Ma XX, Sun DD, Wang S *et al.* Nasal carriage of methicillin-resistant *Staphylococcus aureus* among preclinical medical students: epidemiologic and molecular characteristics of methicillin-resistant *S. aureus* clones. Diagn Microbiol Infect Dis 2011; 70: 22-30.
9. Du J, Chen C, Ding B *et al.* Molecular characterization and antimicrobial susceptibility of nasal *Staphylococcus aureus* isolates from a Chinese medical college campus. PLoS One 2011; 6: e27328.
10. Qu F, Cui E, Guo T *et al.* Nasal colonization of and clonal transmission of methicillin-susceptible *Staphylococcus aureus* among Chinese military volunteers. J Clin Microbiol 2010; 48: 64-69.
11. Brakstad OG, Aasbakk K, Maeland JA. Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the *nuc* gene. J Clin Microbiol 1992; 30: 1654-1660.
12. Harmsen D, Claus H, Witte W *et al.* Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for *spa* repeat determination and database management. J Clin Microbiol 2003; 41: 5442-5448.
13. Lina G, Piemont Y, Godail-Gamot F *et al.* Involvement of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. Clin Infect Dis 1999, 29: 1128-1132.
14. Gorwitz RJ, Kruszon-Moran D, McAllister SK *et al.* (2008) Changes in the prevalence of nasal colonization with *Staphylococcus aureus* in the United States, 2001-2004. J Infect Dis 2008; 197: 1226-1234.
15. Severin JA, Lestari ES, Kuntaman K *et al.* Unusually high prevalence of panton-valentine leukocidin genes among methicillin-sensitive *Staphylococcus aureus* strains carried in the Indonesian population. J Clin Microbiol 2008; 46: 1989-1995.
16. Hamdan-Partida A, Sainz-Espunes T, Bustos-Martinez J. Characterization and persistence of *Staphylococcus aureus* strains isolated from the anterior nares and throats of healthy carriers in a Mexican community. J Clin Microbiol 2010; 48: 1701-1705.
17. den Heijer CD, van Bijnen EM, Paget WJ *et al.* Prevalence and resistance of commensal *Staphylococcus aureus*, including meticillin-resistant *S. aureus*, in nine European countries: a cross-sectional study. Lancet Infect Dis 2013; 13: 409-415.
18. Ateba NU, Schaumburg F, Adegnika AA *et al.* Epidemiology and population structure

of *Staphylococcus aureus* in various population groups from a rural and semi urban area in Gabon, Central Africa. Acta Trop 2012; 124: 42-47.
19. Cole AM, Tahk S, Oren A *et al.* Determinants of *Staphylococcus aureus* nasal carriage. Clin Diagn Lab Immunol 2001; 8: 1064-1069.
20. Eriksen NH, Espersen F, Rosdahl VT, Jensen K. Carriage of *Staphylococcus aureus* among 104 healthy persons during a 19-month period. Epidemiol Infect 1995; 115: 51-60.
21. Kinsman OS, McKenna R, Noble WC. Association between histocompatability antigens (HLA) and nasal carriage of *Staphylococcus aureus*. J Med Microbiol 1983; 16: 215-220.
22. Giarola LB, Dos Santos RR, Bedendo J, da Silva Junior WV, Borelli SD. HLA molecules and nasal carriage of *Staphylococcus aureus* isolated from dialysis and kidney transplant patients at a hospital in Southern Brazil. BMC Res Notes 2012; 5: 90.
23. Zhang YL, Zhou HW, Ma L *et al.* Homology analysis of the epidemiological strains of meticillin-resistant *Staphylococcus aureus* and the strains isolated from the nasal fossa of the medical staff and inpatients. Journal of Southern Medical University 2006; 26: 635-637.
24. Chen H, Liu Y, Jiang X, Chen M, Wang H. Rapid change of methicillin-resistant *Staphylococcus aureus* clones in a Chinese tertiary care hospital over a 15-year period. Antimicrob Agents Chemother 2010; 54: 1842-1847.
25. Zhao C, Liu Y, Zhao M *et al.* Characterization of community acquired *Staphylococcus aureus* associated with skin and soft tissue infection in Beijing: high prevalence of PVL+ ST398. PLoS One 2012; 7: e38577.
26. Chen H, Liu Y, Jiang X, Chen M, Wang H. Rapid change of methicillin-resistant *Staphylococcus aureus* clones in a Chinese tertiary care hospital over a 15-year period. Antimicrob Agents Chemother 2010; 54: 1842-1847.

Chapter 6

Describing the Population Structure of *Staphylococcus aureus* in China and Europe by Multiple-Locus Variable Number Tandem Repeat Analysis; Clues to Geographical Origins of Emergence and Dissemination

Xiaomei Yan#, Leo M. Schouls#, Gerlinde N. Pluister, Xiaoxia Tao, Xiaojie Yu, Jiyuan Yin, Yanyan Song, Shoukui Hu, Fengji Luo, Wanfu Hu, Lihua He, Fanliang Meng, Tjibbe Donker, Eleni Tsompanidou, Jan Maarten van Dijl, Jianzhong Zhang*, Hajo Grundmann*

#These authors contributed equally to this work

Abstract

To compare the genetic population structure of *Staphylococcus aureus* from China and Europe, 1294 human isolates were characterized by multiple-locus variable number tandem repeat analysis (MLVA). In total, MLVA identified 17 MLVA complexes (MCs), comprising 260 MLVA types (MTs) among the Chinese isolates and 372 MTs among the European isolates. The five most frequent MCs among the Chinese isolates belonged to MC398, MC5 subclade a, MC8, MC437 and MC7 and made up 55% of the sample. For the European isolates, the five most frequent MCs consisted of MC5 subclade a, MC45, MC8, MC30 and MC22, which accounted for 64% of the sample. Based on the relative proportions of Chinese versus European isolates among the MC founders and their descendants, MC5 subclade b, MC1933, MC398, MC437, MC621, MC1 and MC123 seem to have originated from China, and MC2, MC22, MC30 and MC45 from Europe. Altogether, this study provides novel hypotheses about the geographical origins of emergence and events leading to the dissemination of different lineages of *S. aureus.*

Introduction

Staphylococcus aureus is a well-known pathogen due to its high virulence potential and its capability of colonizing both humans and domesticated animals. About 20-30% of healthy people living in temperate climates are persistent carriers (43,45). Asymptomatic nasal colonization by *S. aureus* is known to predispose to infection with the same strain (7). *S. aureus* is the most frequent cause of purulent skin and soft tissue infections in humans, but it also has the potential to cause life-threatening blood stream infections, endocarditis, toxic shock syndrome and necrotizing pneumonia.

Compared to other bacterial species, the evolution of *S. aureus* is rather clonal (12). Whole-genome sequences of *S. aureus* strains reveal that core genes, which exist in all isolates, are highly conserved (97%) and diversify mainly by the accumulation of single nucleotide substitutions (21,26,27). In addition to the core genes, more than 700 core variable (CV) genes exist, and the majority encode for surface proteins. Most isolates of *S. aureus* can be grouped into a limited number of clonal lineages or complexes, each of which is defined by highly related core genomes and a unique combination of CV genes. Therefore, it is possible to unequivocally discern most lineages by different molecular typing approaches.

Most molecular typing methods have been developed to unravel the epidemiological relatedness of different isolates for hospital surveillance and/or outbreak investigations. For predominantly clonally evolving species such as *S. aureus*, however, the limited diversity within clonal complexes severely curtails the ability of typing methods to draw micro-epidemiological conclusions, such as the identification of individual transmission events in hospitals. Despite these limitations, molecular methods are able to cluster genetically related isolates if genomic typing data are generated and grouped using distance-based methods. For a sufficiently large and representative number of isolates, a pattern emerges that represents the genetic population structure of that sample, which can provide crucial insights into the abundance of certain lineages among the source populations.

As much attention is focused on the emergence and spread of methicillin-resistant *S. aureus* (MRSA), which has become a frequent source of difficult-to-treat hospital and community-acquired (CA) infections, most of the available strain collections are biased towards MRSA and very little meaningful conclusions can be inferred about natural populations of methicillin-susceptible *S. aureus* (MSSA) from which MRSA lineages happen to emerge. The present study was therefore aimed at describing the genetic structure of large *S. aureus* population samples collected at multiple sites in China and Europe using

comprehensive sampling frames that reflect the natural occurrence of strains and phenotypes.

Materials and methods

Bacterial isolate collection

In order to gather a representative sample of the *S. aureus* population in China, 647 *S. aureus* isolates were collected from both patients and healthy individuals during the years 2005 - 2011. Isolates from patients were collected from two hospitals in eastern China, while healthy carriage isolates were collected from individuals presenting for routine health checks at health centers in the Chaoyang Center for Disease Control and Prevention, Beijing, and the Heilongjiang province Center for Disease Control, Harbin. For comparison, an equivalent number of European *S. aureus* isolates (n=647) were randomly selected from the European survey database of isolates from the Staphylococcal Reference Laboratory (SRL) collected in 2006 and 2007 in 25 countries (16)**.**

Identification and collection of S. aureus isolates

Nasal swabs from healthy carriers were enriched in tryptic soy broth (OXOID, Basingstoke, UK) with 7% NaCl at 37^{o}C for 24 h, and then plated onto mannitol salt agar (OXOID, Basingstoke, UK). Plates were incubated at 37^{o}C for 24 h. Presumptive *S. aureus* colonies were identified by coagulase production using the Slidex Staph Plus kit (Murex Biotech Ltd., Dartford, UK), and confirmed as *S. aureus* by *nuc* PCR (4). The presence of the *mecA* gene was used to define MRSA (4). The clinical isolates were obtained from diagnostic hospital laboratories and were also subjected to *nuc and mecA* PCR for confirmation purposes.

DNA extraction

Two colonies from a plate that had been incubated overnight were suspended in 15 µl TE buffer (10mM Tris-HCl 1mM EDTA PH=8.0) and heated for 10 min at 95ºC. Lysostaphin was added to a final concentration of 100 µg/ml. The samples were incubated for 35 min at 37ºC and then heated for 10 min at 95ºC for enzyme inactivation. Subsequently, 120 µl TE was added and the lysate was used either directly for PCR or stored at -20°C.

MLVA typing

MLVA typing was performed as described by Schouls et al. (38). Briefly, 8 Variable Number Tandem Repeat (VNTR) loci including three coding regions and five non-coding regions were amplified in 2 multiplex PCR reactions. The 4 different fluorescently labelled PCR products were diluted 1:100, mixed with the GeneScan 1200 LIZ size standard (Applied Biosystems,

Foster City, CA, US) and calibrated on an AB 3730 DNA analyzer. The number of the repeats of each VNTR locus was assessed using the GeneMarker software (Softgenetics, State College, PA, US).

Spa tying and multilocus sequence typing (MLST)

All of the isolates were characterized using *spa* typing and representative isolates of MLVA types (MT) were subjected to MLST for the assignment of clonal complexes (CC) (11,18).

pvl gene detection

The Panton-Valentine leukocidin (PVL) genes (*lukS*-PV and *lukF*-PV) were detected by PCR as previously described (25).

Data analysis

Minimum spanning trees were used to illustrate the genetic relationships (Bionumerics, beta version 3.5, Applied Maths, Kortrijk, Belgium). For assignment of MLVA complexes, the entire in-house MLVA database (available at www.mlva.net) was interrogated. MTs were grouped if they differed by no more than a single VNTR whereby the MT with the largest number of single locus variants (SLVs) was regarded as the founder. Groups of 4 or more MTs were regarded as MLVA complexes (MCs). The probability of founders and descendants being Chinese was analyzed by scatter plot. The chi-square statistics (SPSS, SPSS Inc., Chicago, IL, US) was used to test for differences in proportions. A P-value of ≤0.05 was considered statistically significant.

Results

In total, 1294 *S. aureus* were included into the study; 647 were from China, consisting of 253 clinical and 394 healthy carriage isolates (85.5% MSSA, 14.5% MRSA), collected from various locations and sources between 2005 and 2011 (Table 1a). The healthy carriage isolates originated from 1530 and 918 nose swabs that were sampled at health centers in Beijing and Harbin, respectively. For comparison, an equivalent number of European *S. aureus* isolates (n=647, 64.9% MSSA, 35.1% MRSA) were randomly selected from the European survey database (Table 1b).

Table 1a. Overview of 647 Chinese *S. aureus* isolates collected from various locations and sources between 2005 and 2011

Isolate source	Province	No. of isolates	No. of MSSA	No. of MRSA
Patients	Anhui	190	133	57
	Zhejiang	63	32	31
Healthy carriers	Heilongjiang	188	183	5
	Beijing	206	205	1
Total		647	553	94

Table 1b. Overview of 647 European *S. aureus* isolates collected from different countries in 2006-2007.

Isolate source	Country	No. of isolates	No. of MSSA (%)	No. of MRSA (%)
Patients	Austria	49	35(8.3)	14(6.2)
	Belgium	25	3(0.7)	22(9.7)
	Bulgaria	14	6(1.4)	8(3.5)
	Croatia	17	12(2.9)	5(2.2)
	Cyprus	2	2(0.5)	0(0.0)
	Czech Republic	45	28(6.7)	17(7.5)
	Denmark	25	25(5.9)	0(0.0)
	Finland	11	7(1.7)	4(1.8)
	France	71	35(8.3)	36(15.9)
	Germany	47	26(6.2)	21(9.3)
	Greece	3	1(0.2)	2(0.9)
	Hungary	9	8(1.9)	1(0.4)
	Iceland	1	1(0.2)	0(0.0)
	Ireland	57	29(6.9)	28(12.3)
	Italy	37	21(5.0)	16(7.1)
	Netherlands	54	51(12.1)	3(1.3)
	Norway	20	20(4.8)	0(0.0)
	Poland	44	36(8.6)	8(3.6)
	Portugal	32	18(4.3)	14(6.2)
	Slovenia	22	19(4.5)	3(1.3)
	Spain	62	37(8.8)	25(11.0)
Total	-	647	420(100.0)	227(100.0)

MLVA identified 260 MTs and 372 MTs among the Chinese and European isolates,

respectively. Both Chinese and European isolates were clustered into 17 distinct MCs (Fig1A). There was a systematic difference in the distribution of these *S. aureus* complexes between China and Europe (Fig 1A, Table 2). Specifically, the proportion of isolates obtained from Chinese individuals was significantly larger among the eight MLVA complexes MC1, MC5 subclade b, MC7, MC123, MC398, MC437, MC621 and MC1933. In contrast, isolates from European origin dominated five other MCs, namely MC2, MC5 subclade a, MC22, MC30 and MC45. Furthermore, the five most frequently encountered MCs among the Chinese isolates were MC398, MC5 subclade a, MC8, MC437 and MC7, which made up 55.0% of the sample. For the European isolates, the five most frequent MCs were MC5 subclade a, MC45, MC8, MC30 and MC22, which accounted for 64.3% of the sample.

When stratifying by antibiotic resistance type (MSSA vs MRSA), 94 Chinese and 227 European MRSA isolates were divided into 22 MTs and 93 MTs, respectively (Fig 1B). The proportion of the MRSA isolates was significantly larger among four MCs: MC2, MC22, MC8, and MC5 subclade a. Notably, MC2 was composed of MRSA only and all of these isolates originated from Europe. Also, all MRSA isolates in MC22 (76%) were of European origin. Sixty-six percent of all MC8 isolates were MRSA, and 55% of all Chinese and 23% of all European MRSA isolates partitioned into MC8 (Table 2). MC5 subclade a contained most of the remaining MRSA isolates. Interestingly, there was a clear difference in the MT distribution of the Chinese and European isolates in MC8 and MC5 subclade a (Fig S1). The top five MCs of MSSA and MRSA isolates and the corresponding MLST and *spa* types are summarized by country in Tables S1a (MSSA) and S1b (MRSA).

The relative proportions of Chinese versus European isolates among the founders and their descendants were plotted on a two-dimensional scatter plot (Fig 2). MLVA types that are ancestral to each complex are considered to be the founders. An overrepresentation of any geographic region (China or Europe) among both founders and descendants may therefore indicate phylogeographic origin. A clear overrepresentation (proportion >0.5) among both founders and descendants was found for 11 MCs, whereby MC5 subclade b, MC1933, MC398, MC437, MC621, MC1 and MC123 were more likely originating from China, and MC2, MC22, MC30 and MC45 from Europe.

The presence of *pvl* genes was checked for all the Chinese isolates. Thirty-eight isolates were *pvl* positive, which include 5 MRSA and 33 MSSA isolates. Interestingly, 70% (14/20) of the MC5 subclade b isolates were *pvl* positive.

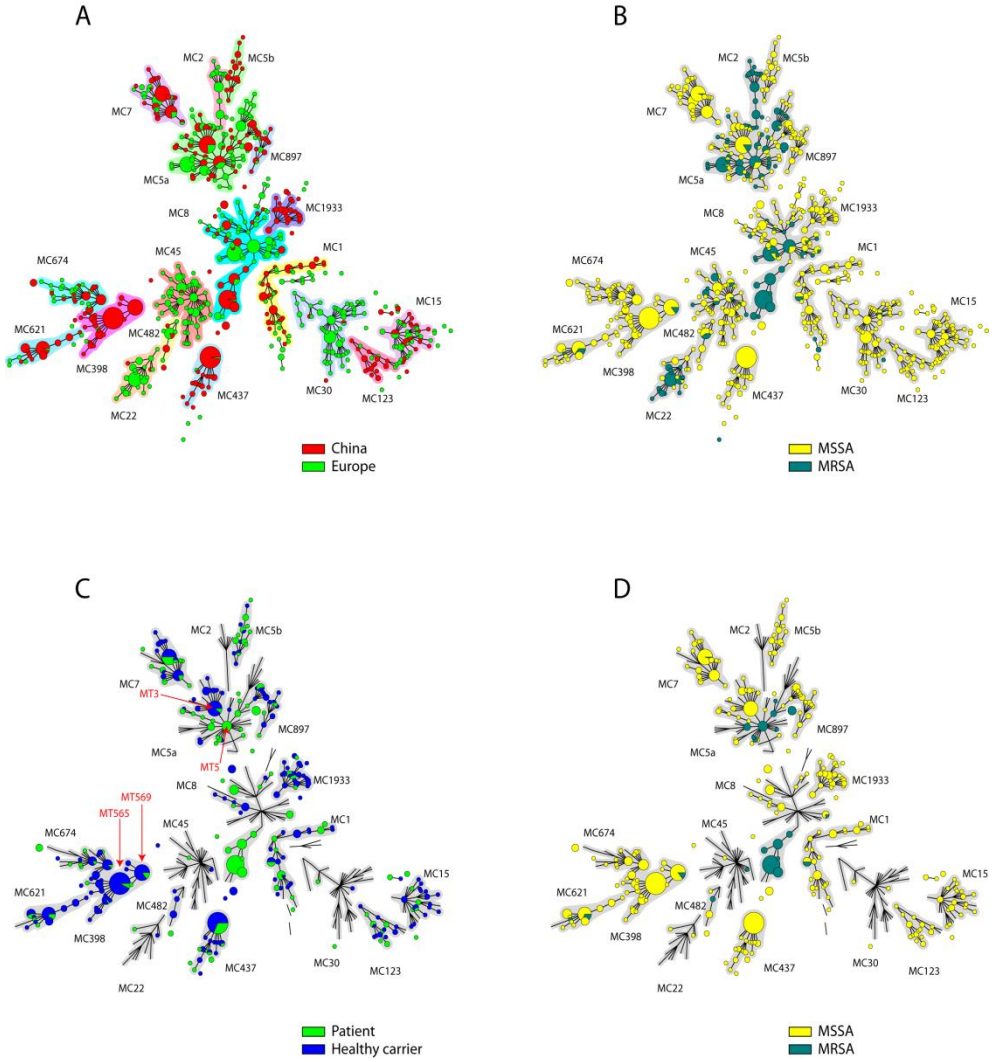

Figure 1. Minimum spanning tree of *S. aureus* isolates from China and Europe based on the relatedness between MLVA types. Each circle represents a single MLVA type, and the size of the circle indicates the number of isolates with the same MLVA type. (A), Geographical origin: China or Europe is indicated in red and green colour code. MLVA complexes are shown by coloured halos. (B), Resistance characteristics: MRSA versus MSSA is indicated in dark green and yellow. (C), Origin of isolates: clinical or carriage for Chinese isolates only. Note that leafless branches indicate the positions of European isolates. (D), Resistance characteristics: MRSA versus MSSA for Chinese isolates only. Note that leafless branches indicate the positions of European isolates.

Table 2. Distribution of MLVA complexes (MCs) of Methicillin-sensitive and resistant *S. aureus* by country

MLVA complex	Clone complex (CC)	Frequency(%) of MSSA			Frequency(%) of MRSA			Frequency(%) overall		
		China	Europe	P-value	China	Europe	P-value	China	Europe	P-value
1	1	38(6.9)	10(2.4)	0.001	3(3.2)	0	0.025	41(6.3)	10(1.5)	<0.0001
2	5	0	0	-	0	28(12.3)	<0.0001	0	28(4.3)	<0.0001
5a	5	58(10.5)	37(8.8)	0.445	20(21.3)	72(31.7)	0.077	78(12.1)	109(16.9)	0.018
5b	88	20(3.6)	0	<0.0001	0	0	-	20(3.1)	0	<0.0001
7	7	52(9.4)	28(6.7)	0.128	1(1.1)	0	0.293	53(8.2)	28(4.3)	0.006
8	8	22(4.0)	33(7.9)	0.011	52(55.3)	54(23.8)	<0.0001	74(11.4)	87(13.4)	0.312
15	15	24(4.3)	33(7.9)	0.027	0	0	-	24(3.7)	33(5.1)	0.278
22	22	2(0.4)	10(2.4)	0.006	0	39(17.2)	<0.0001	2(0.3)	49(7.6)	<0.0001
30	30	2(0.4)	78(18.6)	<0.0001	0	2(0.9)	1	2(0.3)	80(12.4)	<0.0001
45	45	1(0.2)	73(17.4)	<0.0001	1(1.1)	17(7.5)	0.029	2(0.3)	90(13.9)	<0.0001
123	121	21(3.8)	5(1.2)	0.015	0	0	-	21(3.3)	5(0.8)	0.002
398	398	89(16.1)	1(0.2)	<0.0001	3(3.2)	0	0.025	92(14.2)	1(0.1)	<0.0001
437	1	59(10.7)	2(0.5)	<0.0001	0	0	-	59(9.1)	2(0.3)	<0.0001
482	8	5(1.0)	9(2.1)	0.172	2(2.1)	2(0.9)	0.583	7(1.1)	11(1.7)	0.478
621	59	35(6.3)	3(0.7)	<0.0001	6(6.4)	1(0.4)	0.003	41(6.3)	4(0.6)	<0.0001
674	25	29(5.2)	19(4.5)	0.656	0	0	-	29(4.5)	19(2.9)	0.185
897	20	12(2.2)	4(0.9)	0.203	0	0	-	12(1.8)	4(0.6)	0.075
1933	ST6[a]	34(6.2)	0	<0.0001	0	0	-	34(5.3)	0	<0.0001
None		50(9.0)	75(17.9)	-	6(6.4)	12(5.3)	-	56(8.7)	87(13.4)	-
Total		553	420	-	94	227	-	647(100.0)	647(100.0)	-

[a] ST6 is a double locus variant (DLV) of ST5

Discussion

Our present study represents the first attempt to describe two extant populations of *S. aureus* from opposite ends of the same continental shelf - China and Europe. By MLVA typing of isolates obtained through dedicated survey studies, we found that there is a systematic difference in the distribution of *S. aureus* clonal lineages between Europe and China, and we argue that the relative frequencies of Chinese versus European isolates among founders and descendants point to different geographic origins of several lineages.

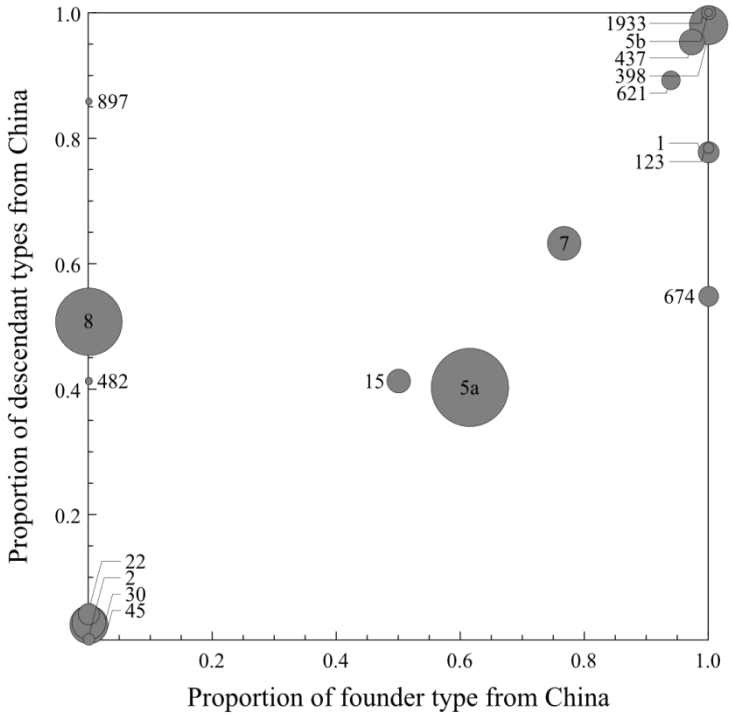

Figure 2. The proportion of isolates of the *S. aureus* founder types from China and the proportion of isolates of descendant types from China in each MLVA complex. Each circle represents a MLVA complex (indicated by the MC number) with the circle size indicating the respective number of isolates. The MLVA complexes in the top right of the graph more likely originate from China, while the complexes at the left bottom more likely originate from Europe.

It should be noted that there are clear limitations to this study, mainly due to unequal sampling. The Chinese *S. aureus* sample was obtained from two groups, whereby the majority of isolates (n=394) were from healthy carriers while only 253 were clinical isolates. This is in stark contrast to the European sample, which was entirely made up of clinical isolates. This explains the larger proportion of MRSA in the European sample. Nevertheless, we argue that clinical samples are representative of carriage isolates as there has so far been no evidence to the contrary. Indeed, it has been shown that 80% of the patients, who developed *S. aureus* infection after admission to a hospital, were infected by their own colonizing strains (44,46). Moreover, a recent study carried out among healthy carriers in

nine European nations (8) revealed a frequency distribution of the main lineages among MSSA and MRSA that was almost indistinguishable from the frequency distribution of the European sample used in our present study. It can thus be assumed that the spectrum and diversity of MRSA and MSSA reflect the natural distribution of lineages in Europe despite the enrichment of nosocomial MRSA in the European sample.

Another incongruence that deserves consideration relates to the geodemographic representativeness of the samples used in this study. The Chinese isolates were collected in far fewer locations than the European isolates (Table 1a and 1b). However, healthy carriers sampled in Beijing were from 31 of the 34 provincial level divisons of China and included, apart from ethnic Han Chinese, also individuals from 16 of the 55 other ethnic minorities in China. This shows that the population snapshot from Beijing was hardly local, but rather representative for a much larger geographical region. Furthermore, a recent study on the diversity of *S. aureus* bacteraemia isolates from 16 hospitals in 12 cities in China showed a similar distribution of lineages, whereby ST239 and ST5 were the main MRSA genotypes, while CC7, CC188, CC5 and CC398 were the dominant MSSA genotypes (20). We therefore believe that the results of our present study were not biased to the extent that it would invalidate the overall conclusions, which can highlight the differences in the distribution of major clonal linaeges in China and Europe. This difference was particularly pronounced for four international clones (MC2, MC22, MC30 and MC45) that are dominant in Europe, and for successful hospital-acquired (HA-)MRSA with European-wide distribution (MC2, MC5 subclade a, MC8 and MC22). Compared to the European isolates, eight MLVA complexes (MC1, MC7, MC123, MC398, MC437, MC621, MC1933 and MC5 subclade b) were found to be dominant among the Chinese isolates. Altogether, the present clustering based on minimum spanning trees and scatter plot analysis suggests that several of the major clonal lineages here identified have a different ancestral origin whereby some are more likely to originate from East-Asia while others are more likely to have emerged in Europe.

The comparison with the European isolates suggests that the Chinese sample consists of a combination of pandemic lineages and a considerable number of local clones. MC8/ST239/CC8 and MC5/ST5/CC5 are clear representatives of pandemic MRSA lineages in China, which were also shown in previous studies (6,28,50). Only a few healthy carrier isolates were found to belong to MC8/ST239/CC8, whereas most of such isolates were obtained from clinical samples, which support the view that this is a particularly successful lineage in hospitals (Fig 1C). This idea is further supported by the identification of a novel surface protein named SasX in ST239 isolates, which could represent a “driving force” for the

MRSA epidemic in Chinese hospitals (24). The minimum spanning tree shows that the founder of MC8 consists entirely of European isolates, while all the Chinese isolates in this MC represent descendants (Fig1, A and D). This finding fits with the evolutionary model, which argues that the ST239-MRSA-III lineage evolved from a most recent, common ancestor in Europe (39). Whole-genome sequencing also revealed a single introduction of ST239 into South America, followed by a clonal expansion, and subsequent diversification of the ST239 lineage (19).

MC5 represents another pandemic lineage in China. The MC5 subclade a corresponds to CC5, which is a common and widespread clonal complex. Minimum spanning tree and scatter plot analyses suggest that this lineage may have emerged neither in Europe nor in China. Instead, it appears to diversify along different trajectories caused by geographic segregation. This hypothesis was supported by investigating the evolutionary history of ST5-MRSA, which showed that SCC*mec* acquisition occurs frequently in local *S. aureus* populations, while the long-distance geographical spread of MRSA is relatively rare (35). MC5 subclade b is a Chinese MSSA clade and most of the respective isolates are PVL positive. MLST analysis of this clade showed that all of these isolates belong to two novel sequence types, namely ST2148 and ST2141, which are both single locus variants (SLV) of ST88 (CC88). ST88 was reported to spread over a large geographical area in Africa (33). This clonal lineage was also frequently isolated in Australia and sporadically reported in Europe (32). Our present scatter plot and PVL analyses suggest that the MC5 subclade b may have originated from China.

MSSA MC398/ST398/CC398 represented the most frequently identified human colonizer in China, encompassing 16% of all MSSA isolates of the Chinese sample. Consistent with this observation, ST398/CC398 MSSA strains have also been reported previously as major causative agents for HA-MSSA and CA-MSSA infections in China, and they were frequently found to be carried by healthy Chinese children from different regions (6,48,52) . Our own previous observations also showed that isolates of this lineage can be retrieved from slaughter pigs in the Heilongjiang province (51). In Europe, the ST398-MRSA lineage is mainly associated with livestock, and it is occasionally transmitted to humans during occupational exposure (34). It has furthermore been suggested that livestock-associated (LA)-ST398 MRSA is mostly transmitted among veterinarians and their families rather than being transmitted in hospitals (2,3,41). In contrast, the ST398 MSSA clone found in China seems to be readily transmitted between humans, both in hospitals and in the community (52). In the present study, we found two major MTs belonging to MC398, namely

MT565/MC398/ST398/CC398/t571 and MT569/MC398/ST398/CC398/t034 (Fig1C). Interestingly, MT569 isolates were also identified in pigs (data not shown), while MT565 isolates were so far exclusively isolated from humans. This finding suggests that these two main subclones of MC398 in China may have specifically adapted to different hosts. In this context it is noteworthy that in recent years an increasing number of ST398/CC398/t571 MSSA infections have been reported in geographically diverse regions around the globe (1,36,42). It still remains to be seen if these isolates are monophyletic and represent a public health challenge in Europe and China.

MC621 was another complex which likely originated from and disseminated in China. One of the SLVs of MT621/MC621/ST2147/t437, which was identified as the founder of this complex, is MT1035/MC621/ST59/t437. E-burst analysis showed that ST2147 does not belong to any known clonal complex. Different lineages occasionally share the same *spa* gene indicative of homoplasy within the *spa* region. MRSA isolates belonging to ST59/CC59 were previously identified as the most common type of CA-MRSA in Chinese children with skin and soft-tissue infections (SSTIs) (49). Importantly, ST59/CC59/t437 was also reported as the predominant CA-MRSA type in other Asian countries (13,14,40), and it was recently identified across Europe (15). Whether this predominant CA-MRSA clone has evolved from MC621/ST2147/t437 still needs to be elucidated. It remains also to be seen whether the *spa* type t437 confers an advantage in terms of transmissibility or tenacity, which would explain the abundance of this *spa* type among community-acquired MRSA in China.

Some lineages, such as MC2, MC22, MC30 and MC45, which represent successful clones in Europe hardly exist in China. MC2 is related to the Southern-German HA-MRSA clone (ST228/CC5), which was reported in Southern Germany and Italy in the mid-1990s (29,47), and which has spread through the Alpine, Balkan and Appenine regions in recent years (32). MC22/ST22/CC22 is the most successful European MRSA clone. One reason for its apparent absence from China could be that MC22/ST22/CC22 did not yet have sufficient time to diffuse in China as it did in Europe where it emerged as EMRSA-15 in the late 1980s (22). Furthermore, MC30/CC30 and MC45/CC45 were only found in the European sample. This finding is in accordace with previous studies, where the two complexes were confined to Europe and the US (30,31,37).

Lastly, our scatter plot data suggest that several MCs (MC7/ST7/CC7, MC15/ST15/CC15, MC482/ST72/CC8, MC674/ST25/CC25 and MC897/ST20/CC20) may neither have originated from Europe nor China. Consistent with this view, MC7/ST7/CC7, MC15/ST15/CC15 and MC674/ST25/CC25 MSSA have been reported as world-wide

successful MSSA lineages (5,9,10). Furthermore, MC482/ST72/CC8 is the main clone of CA-MRSA infections in Korea and it has been reported in children adopted to Europe from South Korea (17,23). Whole-genome sequencing should be performed to further elucidate the evolution of these clones.

In conclusion, the present study provides novel hypotheses about the geographical origins of emergence and events leading to the dissemination of clonal lineages of *S. aureus* with particular public health importance in China and Europe. As such the results will serve as leads for future studies to better understand the evolution of the *S. aureus* species as a whole.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant No. 81301463), by the State Key Laboratory for Infectious Disease Prevention and Control Fund of China (2012SKLID202), and by an educational grant from the Graduate School of Medical Sciences of the University of Groningen, the Netherlands.

Supplementary Materials

Supplementary Figure 1. Minimum spanning tree displaying the MRSA/MSSA distribution among Chinese and European isolates belonging to MC8 and MC5a.

Supplementary Table 1a. The top five MCs and their MLST and spa types of MSSA by country.

Supplementary Table 1b. The top five MCs and their MLST and *spa* types of MRSA by country.

Reference List

1. Bhat, M., C. Dumortier, B. S. Taylor, M. Miller, G. Vasquez, J. Yunen, K. Brudney, E. Sanchez, C. Rodriguez-Taveras, R. Rojas, P. Leon, and F. D. Lowy. 2009. Staphylococcus aureus ST398, New York City and Dominican Republic. Emerg.Infect.Dis. 15:285-7.
2. Bootsma, M. C., M. W. Wassenberg, P. Trapman, and M. J. Bonten. 2011. The nosocomial transmission rate of animal-associated ST398 meticillin-resistant *Staphylococcus aureus*. J.R.Soc.Interface 8:578-84.
3. Bosch, T., E. Verkade, L. M. van, F. Landman, J. Kluytmans, and L. M. Schouls. 2015. Transmission and persistence of livestock-associated MRSA among veterinarians and their household members. Appl.Environ.Microbiol. 81:124-9.
4. Brakstad, O. G., K. Aasbakk, and J. A. Maeland. 1992. Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the nuc gene. J.Clin.Microbiol. 30:1654-60.
5. Breurec, S., C. Fall, R. Pouillot, P. Boisier, S. Brisse, F. Diene-Sarr, S. Djibo, J. Etienne, M. C. Fonkoua, J. D. Perrier-Gros-Claude, C. E. Ramarokoto, F. Randrianirina, J. M. Thiberge, S. B. Zriouil, B. Garin, and F. Laurent. 2011. Epidemiology of methicillin-susceptible *Staphylococcus aureus* lineages in five major African towns: high prevalence of Panton-Valentine leukocidin genes. Clin.Microbiol.Infect. 17:633-9.
6. Chen, H., Y. Liu, X. Jiang, M. Chen, and H. Wang. 2010. Rapid change of methicillin-resistant *Staphylococcus aureus* clones in a Chinese tertiary care hospital over a 15-year period. Antimicrob.Agents Chemother. 54:1842-7.
7. DeLeo, F. R., M. Otto, B. N. Kreiswirth, and H. F. Chambers. 2010. Community-associated meticillin-resistant *Staphylococcus aureus*. Lancet 375:1557-68.
8. den Heijer, C. D., E. M. van Bijnen, W. J. Paget, M. Pringle, H. Goossens, C. A. Bruggeman, F. G. Schellevis, and E. E. Stobberingh. 2013. Prevalence and resistance of commensal *Staphylococcus aureus*, including meticillin-resistant *S. aureus*, in nine European countries: a cross-sectional study. Lancet Infect.Dis. 13:409-15.
9. Deurenberg, R. H. and E. E. Stobberingh. 2008. The evolution of *Staphylococcus aureus*. Infect.Genet.Evol. 8:747-63.
10. Donker, G. A., R. H. Deurenberg, C. Driessen, S. Sebastian, S. Nys, and E. E. Stobberingh. 2009. The population structure of *Staphylococcus aureus* among general practice patients from The Netherlands. Clin.Microbiol.Infect. 15:137-43.
11. Enright, M. C., N. P. Day, C. E. Davies, S. J. Peacock, and B. G. Spratt. 2000. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. J.Clin.Microbiol. 38:1008-15.
12. Feil, E. J., J. E. Cooper, H. Grundmann, D. A. Robinson, M. C. Enright, T. Berendt, S. J. Peacock, J. M. Smith, M. Murphy, B. G. Spratt, C. E. Moore, and N. P. Day. 2003. How clonal is *Staphylococcus aureus*? J.Bacteriol. 185:3307-16.
13. Geng, W., Y. Yang, D. Wu, G. Huang, C. Wang, L. Deng, Y. Zheng, Z. Fu, C. Li, Y. Shang, C. Zhao, S. Yu, and X. Shen. 2010. Molecular characteristics of community-acquired, methicillin-resistant *Staphylococcus aureus* isolated from Chinese children. FEMS Immunol.Med.Microbiol. 58:356-62.
14. Geng, W., Y. Yang, D. Wu, W. Zhang, C. Wang, Y. Shang, Y. Zheng, L. Deng, Z. Fu, X. Li, S. Yu, and X. Shen. 2010. Community-acquired, methicillin-resistant *Staphylococcus aureus* isolated from children with community-onset pneumonia in China. Pediatr.Pulmonol. 45:387-94.

15. Glasner, C., G. Pluister, H. Westh, J. P. Arends, J. Empel, E. Giles, F. Laurent, F. Layer, L. Marstein, A. Matussek, A. Mellmann, M. Perez-Vasquez, E. Ungvari, X. Yan, H. Zemlickova, H. Grundmann, and J. M. van Dijl. 2015. *Staphylococcus aureus spa* type t437: identification of the most dominant community-associated clone from Asia across Europe. Clin.Microbiol.Infect. 21:163-8.
16. Grundmann, H., D. M. Aanensen, C. C. van den Wijngaard, B. G. Spratt, D. Harmsen, and A. W. Friedrich. 2010. Geographic distribution of *Staphylococcus aureus* causing invasive infections in Europe: a molecular-epidemiological analysis. PLoS.Med. 7:e1000215.
17. Gustafsson, E. B., H. Ringberg, and P. J. Johansson. 2007. MRSA in children from foreign countries adopted to Swedish families. Acta Paediatr. 96:105-8.
18. Harmsen, D., H. Claus, W. Witte, J. Rothganger, H. Claus, D. Turnwald, and U. Vogel. 2003. Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for *spa* repeat determination and database management. J.Clin.Microbiol. 41:5442-8.
19. Harris, S. R., E. J. Feil, M. T. Holden, M. A. Quail, E. K. Nickerson, N. Chantratita, S. Gardete, A. Tavares, N. Day, J. A. Lindsay, J. D. Edgeworth, L. H. de, J. Parkhill, S. J. Peacock, and S. D. Bentley. 2010. Evolution of MRSA during hospital transmission and intercontinental spread. Science 327:469-74.
20. He, W., H. Chen, C. Zhao, F. Zhang, H. Li, Q. Wang, X. Wang, and H. Wang. 2013. Population structure and characterisation of *Staphylococcus aureus* from bacteraemia at multiple hospitals in China: association between antimicrobial resistance, toxin genes and genotypes. Int.J.Antimicrob.Agents. 42:211-9.
21. Holden, M. T., E. J. Feil, J. A. Lindsay, S. J. Peacock, N. P. Day, M. C. Enright, T. J. Foster, C. E. Moore, L. Hurst, R. Atkin, A. Barron, N. Bason, S. D. Bentley, C. Chillingworth, T. Chillingworth, C. Churcher, L. Clark, C. Corton, A. Cronin, J. Doggett, L. Dowd, T. Feltwell, Z. Hance, B. Harris, H. Hauser, S. Holroyd, K. Jagels, K. D. James, N. Lennard, A. Line, R. Mayes, S. Moule, K. Mungall, D. Ormond, M. A. Quail, E. Rabbinowitsch, K. Rutherford, M. Sanders, S. Sharp, M. Simmonds, K. Stevens, S. Whitehead, B. G. Barrell, B. G. Spratt, and J. Parkhill. 2004. Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. Proc.Natl.Acad.Sci.U.S.A 101:9786-91.
22. Holden, M. T., L. Y. Hsu, K. Kurt, L. A. Weinert, A. E. Mather, S. R. Harris, B. Strommenger, F. Layer, W. Witte, L. H. de, R. Skov, H. Westh, H. Zemlickova, G. Coombs, A. M. Kearns, R. L. Hill, J. Edgeworth, I. Gould, V. Gant, J. Cooke, G. F. Edwards, P. R. McAdam, K. E. Templeton, A. McCann, Z. Zhou, S. Castillo-Ramirez, E. J. Feil, L. O. Hudson, M. C. Enright, F. Balloux, D. M. Aanensen, B. G. Spratt, J. R. Fitzgerald, J. Parkhill, M. Achtman, S. D. Bentley, and U. Nubel. 2013. A genomic portrait of the emergence, evolution, and global spread of a methicillin-resistant *Staphylococcus aureus* pandemic. Genome Res. 23:653-64.
23. Kim, E. S., J. S. Song, H. J. Lee, P. G. Choe, K. H. Park, J. H. Cho, W. B. Park, S. H. Kim, J. H. Bang, D. M. Kim, K. U. Park, S. Shin, M. S. Lee, H. J. Choi, N. J. Kim, E. C. Kim, M. D. Oh, H. B. Kim, and K. W. Choe. 2007. A survey of community-associated methicillin-resistant *Staphylococcus aureus* in Korea. J Antimicrob.Chemother. 60:1108-14.
24. Li, M., X. Du, A. E. Villaruz, B. A. Diep, D. Wang, Y. Song, Y. Tian, J. Hu, F. Yu, Y. Lu, and M. Otto. 2012. MRSA epidemic linked to a quickly spreading colonization and virulence determinant. Nat.Med. 18:816-9.
25. Lina, G., Y. Piemont, F. Godail-Gamot, M. Bes, M. O. Peter, V. Gauduchon, F. Vandenesch, and J. Etienne. 1999. Involvement of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. Clin.Infect.Dis. 29:1128-32.
26. Lindsay, J. A. 2010. Genomic variation and evolution of *Staphylococcus aureus*.

Int.J.Med.Microbiol. 300:98-103.
27. Lindsay, J. A. and M. T. Holden. 2006. Understanding the rise of the superbug: investigation of the evolution and genomic variation of *Staphylococcus aureus*. Funct.Integr.Genomics 6:186-201.
28. Liu, Y., H. Wang, N. Du, E. Shen, H. Chen, J. Niu, H. Ye, and M. Chen. 2009. Molecular evidence for spread of two major methicillin-resistant *Staphylococcus aureus* clones with a unique geographic distribution in Chinese hospitals. Antimicrob.Agents Chemother. 53:512-8.
29. Mato, R., F. Campanile, S. Stefani, M. I. Crisostomo, M. Santagati, S. I. Sanches, and L. H. de. 2004. Clonal types and multidrug resistance patterns of methicillin-resistant *Staphylococcus aureus* (MRSA) recovered in Italy during the 1990s. Microb.Drug Resist. 10:106-13.
30. Melles, D. C., R. F. Gorkink, H. A. Boelens, S. V. Snijders, J. K. Peeters, M. J. Moorhouse, P. J. van der Spek, W. B. van Leeuwen, G. Simons, H. A. Verbrugh, and B. A. van. 2004. Natural population dynamics and expansion of pathogenic clones of *Staphylococcus aureus*. J.Clin.Invest 114:1732-40.
31. Melles, D. C., F. C. Tenover, M. J. Kuehnert, H. Witsenboer, J. K. Peeters, H. A. Verbrugh, and B. A. van. 2008. Overlapping population structures of nasal isolates of *Staphylococcus aureus* from healthy Dutch and American individuals. J.Clin.Microbiol. 46:35-241.
32. Monecke, S., G. Coombs, A. C. Shore, D. C. Coleman, P. Akpaka, M. Borg, H. Chow, M. Ip, L. Jatzwauk, D. Jonas, K. Kadlec, A. Kearns, F. Laurent, F. G. O'Brien, J. Pearson, A. Ruppelt, S. Schwarz, E. Scicluna, P. Slickers, H. L. Tan, S. Weber, and R. Ehricht. 2011. A field guide to pandemic, epidemic and sporadic clones of methicillin-resistant *Staphylococcus aureus*. PLoS One 6:e17936.
33. Moremi, N., S. E. Mshana, E. Kamugisha, J. Kataraihya, D. Tappe, U. Vogel, E. F. Lyamuya, and H. Claus. 2012. Predominance of methicillin resistant *Staphylococcus aureus* -ST88 and new ST1797 causing wound infection and abscesses. J.Infect.Dev.Ctries. 6:620-5.
34. Morgan, M. 2008. Methicillin-resistant *Staphylococcus aureus* and animals: zoonosis or humanosis? J.Antimicrob.Chemother. 62:1181-7.
35. Nubel, U., P. Roumagnac, M. Feldkamp, J. H. Song, K. S. Ko, Y. C. Huang, G. Coombs, M. Ip, H. Westh, R. Skov, M. J. Struelens, R. V. Goering, B. Strommenger, A. Weller, W. Witte, and M. Achtman. 2008. Frequent emergence and limited geographic dispersal of methicillin-resistant *Staphylococcus aureus*. Proc.Natl.Acad.Sci.U.S.A 105:14130-5.
36. Rasigade, J. P., F. Laurent, G. Lina, H. Meugnier, M. Bes, F. Vandenesch, J. Etienne, and A. Tristan. 2010. Global distribution and evolution of Panton-Valentine leukocidin-positive methicillin-susceptible *Staphylococcus aureus*, 1981-2007. J.Infect.Dis. 201:1589-97.
37. Sakwinska, O., G. Kuhn, C. Balmelli, P. Francioli, M. Giddey, V. Perreten, A. Riesen, F. Zysset, D. S. Blanc, and P. Moreillon. 2009. Genetic diversity and ecological success of *Staphylococcus aureus* strains colonizing humans. Appl.Environ.Microbiol. 75:175-83.
38. Schouls, L. M., E. C. Spalburg, L. M. van, X. W. Huijsdens, G. N. Pluister, M. G. van Santen-Verheuvel, H. G. van der Heide, H. Grundmann, M. E. Heck, and A. J. de Neeling. 2009. Multiple-locus variable number tandem repeat analysis of *Staphylococcus aureus*: comparison with pulsed-field gel electrophoresis and *spa*-typing. PLoS.One. 4:e5082.
39. Smyth, D. S., L. K. McDougal, F. W. Gran, A. Manoharan, M. C. Enright, J. H. Song, L. H. de, and D. A. Robinson. 2010. Population structure of a hybrid clonal group of methicillin-resistant *Staphylococcus aureus*, ST239-MRSA-III. PLoS.One. 5:e8582.
40. Song, J. H., P. R. Hsueh, D. R. Chung, K. S. Ko, C. I. Kang, K. R. Peck, J. S. Yeom,

S. W. Kim, H. H. Chang, Y. S. Kim, S. I. Jung, J. S. Son, T. M. So, M. K. Lalitha, Y. Yang, S. G. Huang, H. Wang, Q. Lu, C. C. Carlos, J. A. Perera, C. H. Chiu, J. W. Liu, A. Chongthaleong, V. Thamlikitkul, and P. H. Van. 2011. Spread of methicillin-resistant *Staphylococcus aureus* between the community and the hospitals in Asian countries: an ANSORP study. J.Antimicrob.Chemother. 66:1061-9.

41. van Cleef, B. A., E. J. Verkade, M. W. Wulf, A. G. Buiting, A. Voss, X. W. Huijsdens, P. W. van, M. N. Mulders, and J. A. Kluytmans. 2010. Prevalence of livestock-associated MRSA in communities with high pig-densities in The Netherlands. PLoS.One. 5:e9385.
42. van, B. A., D. C. Melles, J. K. Peeters, W. B. van Leeuwen, D. E. van, X. W. Huijsdens, E. Spalburg, A. J. de Neeling, and H. A. Verbrugh. 2008. Methicillin-resistant and -susceptible *Staphylococcus aureus* sequence type 398 in pigs and humans. Emerg.Infect.Dis. 14:479-83.
43. van, B. A., N. J. Verkaik, C. P. de Vogel, H. A. Boelens, J. Verveer, J. L. Nouwen, H. A. Verbrugh, and H. F. Wertheim. 2009. Reclassification of *Staphylococcus aureus* nasal carriage types. J.Infect.Dis. 199:1820-6.
44. von, E. C., K. Becker, K. Machka, H. Stammer, and G. Peters. 2001. Nasal carriage as a source of *Staphylococcus aureus* bacteremia. Study Group. N.Engl.J.Med. 344:11-6.
45. Wertheim, H. F., D. C. Melles, M. C. Vos, L. W. van, B. A. van, H. A. Verbrugh, and J. L. Nouwen. 2005. The role of nasal carriage in *Staphylococcus aureus* infections. Lancet Infect.Dis. 5:751-62.
46. Wertheim, H. F., M. C. Vos, A. Ott, B. A. van, A. Voss, J. A. Kluytmans, P. H. van Keulen, C. M. Vandenbroucke-Grauls, M. H. Meester, and H. A. Verbrugh. 2004. Risk and outcome of nosocomial *Staphylococcus aureus* bacteraemia in nasal carriers versus non-carriers. Lancet 364:703-5.
47. Wisplinghoff, H., T. Bischoff, S. M. Tallent, H. Seifert, R. P. Wenzel, and M. B. Edmond. 2004. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clin.Infect.Dis. 39:309-17.
48. Wu, D., X. Li, Y. Yang, Y. Zheng, C. Wang, L. Deng, L. Liu, C. Li, Y. Shang, C. Zhao, S. Yu, and X. Shen. 2011. Superantigen gene profiles and presence of exfoliative toxin genes in community-acquired meticillin-resistant *Staphylococcus aureus* isolated from Chinese children. J.Med.Microbiol. 60:35-45.
49. Wu, D., Q. Wang, Y. Yang, W. Geng, Q. Wang, S. Yu, K. Yao, L. Yuan, and X. Shen. 2010. Epidemiology and molecular characteristics of community-associated methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* from skin/soft tissue infections in a children's hospital in Beijing, China. Diagn.Microbiol.Infect.Dis. 67:1-8.
50. Xu, B. L., G. Zhang, H. F. Ye, E. J. Feil, G. R. Chen, X. M. Zhou, X. M. Zhan, S. M. Chen, and W. B. Pan. 2009. Predominance of the Hungarian clone (ST 239-III) among hospital-acquired meticillin-resistant *Staphylococcus aureus* isolates recovered throughout mainland China. J.Hosp.Infect. 71:245-55.
51. Yan, X., X. Yu, X. Tao, J. Zhang, B. Zhang, R. Dong, C. Xue, H. Grundmann, and J. Zhang. 2014. *Staphylococcus aureus* ST398 from slaughter pigs in northeast China. Int.J.Med.Microbiol. 304:379-83.
52. Zhao, C., Y. Liu, M. Zhao, Y. Liu, Y. Yu, H. Chen, Q. Sun, H. Chen, W. Jiang, Y. Liu, S. Han, Y. Xu, M. Chen, B. Cao, and H. Wang. 2012. Characterization of community acquired *Staphylococcus aureus* associated with skin and soft tissue infection in Beijing: high prevalence of PVL+ ST398. PLoS.One. 7:e38577.

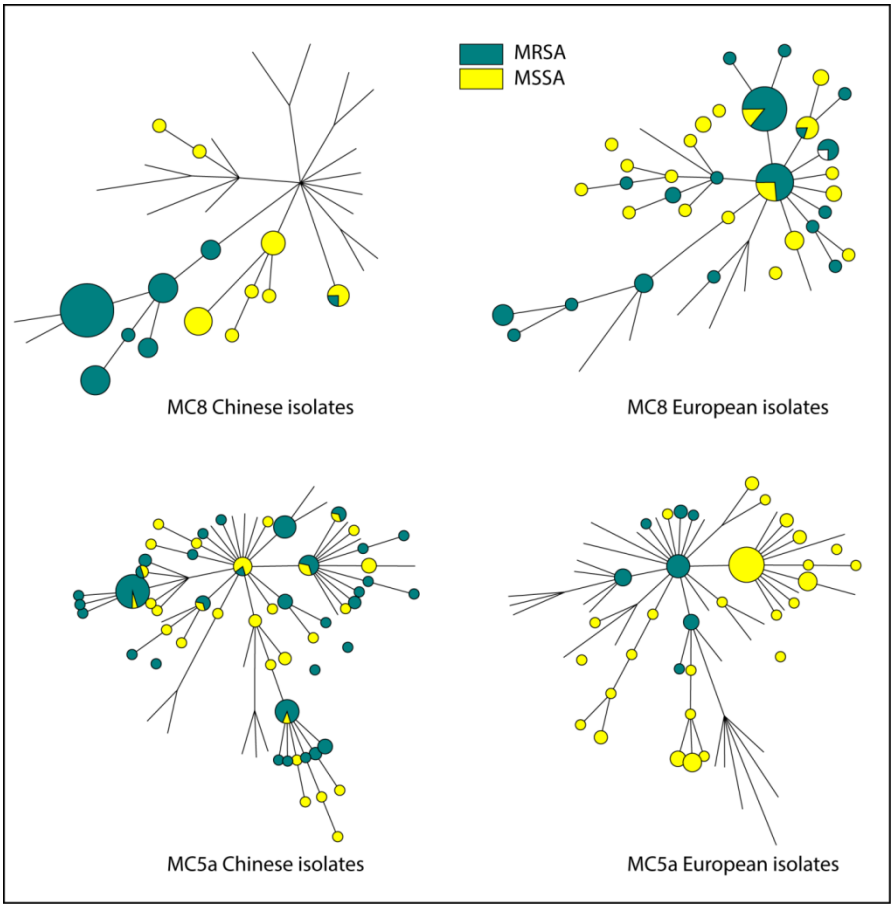

Figure S1. Minimum spanning tree displaying the MRSA/MSSA distribution among Chinese and European isolates belonging to MC8 and MC5a. Each circle represents a single MLVA type, and the size of the circle indicates the number of isolates with the same MLVA type. Resistance characteristics: MRSA versus MSSA are indicated in dark green and yellow colour code. Note that branches without nodes indicate MLVA-types that are absent from either the Chinese or European isolates.

Table S1a. The top five MCs and their MLST and *spa* types of MSSA by country.

China						Europe					
Rank	MLVA complex (No. of isolates)	No. of MLVA types	Clone complex (CC)	MLST (No. of isolates)	*spa* (No.of isolates)	Rank	MLVA complex (No.of isolates)	No. of MLVA types	Clone complex (CC)	MLST(No.of isolates)	*spa*(No.of isolates)
1	398(89)	19	CC398	ST398(78)	t571(47),t034(22), t1451(8),t2370(1)	1	30(78)	51	CC30	ST30(27)	t012(19),t021(8)
			CC398	ST2140(6)	t011(4),t8099(2)				CC30	(ST34)(7)	t166(7)
			CC398	ST2392(2)	t1250(2)				CC8	ST239(1)	t037(1)
			n.d.	n.d.(3)	n.t.(3)				CC7	ST7(1)	t091(1)
									CC30	(ST-30, ST-38)(2)	t018(2)
									CC30	ST30(1)	t019(1)
									CC30	(ST39)(3)[a]	t382(3)
									n.d.	n.d.(36)	t017(2),t122(2),t1414(2),t2271(2),t046(1),t089(1),t1051(1),t1192(1),t129(1),t136(1),t1515(1),t153(1),t1642(1),t1654(1),t1978(1),t2018(1),t2243(1),t2270(1),t2359(1),t2408(1),t2506(1),t251(1),t2585(1),t2602(1),t2702(1),t342(1),t399(1),t433(1),t440(1),t685(1),t767(1),t840(1)

2	437(59)	16	CC1	ST2139(55)	t189(55)	2	45(73)	43	CC45	ST45(19)	t015(16),t050(3)
			CC188	ST2393	t416(1)				CC45	(ST45,ST47)(9)	t026(9)
			n.d.	n.d.(3)	t2612(2),t8916(1)				CC45	(ST45)(7)	t230(7)
									CC45	(ST45,ST46)(6)	t065(6)
									n.d.	n.d.(32)	t073(1),t095(2),t116(1),t130(2),,t2053(1),t2135(1),t2223(1),t2245(1),t2256(1),t2286(1),t2301(1),t2383(1),t2539(1),t2709(1),t2727(1),t282(1),t2940(1),t2946(1),t302(2),t331(2),t505(1),t576(1),t620(1),t630(2),t728(1),t772(1),t779(1)
3	5a(58)	26	CC5	ST5(29)	t002(28),t105(1)	3	5a(37)		CC5	ST5,ST231(14)	t002(14)
			CC5	ST2144(6)	t548(6)				CC5	ST-5,ST125(3)	t067(3)
			CC5	ST2115(1)	t1107(1)				CC5	(ST5)(8)	t053(2),t105(2),t179(2),t311(2)
			CC5	ST2394(1)	t4867(1)				CC5	ST225(1)	t003(1)
			CC5	ST965(1)	t9099(1)				CC80	ST80(1)	t045(1)
			CC5	(ST-5, ST-225)(2)	t045(2)				n.d.	n.d.(9)	t088(1),t1400(1),t1567(1),t2051(1),t2587(1),t447(1),t548(2),t777(1)
			CC5	(ST-5)(2)	t179(1),t311(1)						

			n.d.	n.d.(16)	t568(4),t1305(3), t1062(2),t1393(2), t640(2),t1265(1), t8918(1),t9110(1)						
4	7(52)	18	CC7	ST7(48)	t796(27),t091(21)	4	15(33)	23	CC15	ST15(16)	t084(16)
			n.d.	n.d.(4)	t7710(2),t1119(1),t8927(1)				CC15	(ST15,ST620)(2)	t346(2)
									CC15	(ST15)(1)	t085(1)
									n.d.	n.d.(14)	t1034(1),t1877(1),t2055(1),t2296(1),t2325(1),t2452(1),t254(1),t2782(1),t279(1),t2949(1),t335(1),t593(1),t645(1),t853(1)
5	1(38)	21	CC1	ST401(34)	t127(29),t286(5)	5	8(33)	20	CC8	ST8(19)	t008(15),t024(4)
			CC1	ST2143(1)	t3471(1)				CC8	(ST8)(2)	t064(2)
			CC1	ST1(1)	t768(1)				CC7	ST7(1)	t091(1)
			CC1	(ST1)(2)	t386(2)				n.d.	n.d.(11)	t121(1),t1391(1),t150(1),t2140(1),t2229(1),t2374(1),t2420(1),t2450(1),t2705(1),t334(1),t4367(1)

n.d.; not determined; n.t.; not typeable; a. Mellmann A, 2008

Table S1b. The top five MCs and their MLST and *spa* types of MRSA by country.

China						Europe					
Rank	MLVA complex (No. of isolates)	No. of MLVA types	Clone complex (CC)	MLST (No. of isolates)	*spa* (No. of isolates)	Rank	MLVA complex (No. of isolates)	No. of MLVA types	Clone complex (CC)	MLST (No. of isolates)	*spa* (No. of isolates)
1	8(52)	7	CC8	ST239(50)	t030(27),t037(23)	1	5a(72)	29	CC5	ST5,ST231(23)	t002(22),t045(1)
			n.d.	n.d.(2)	t5554(1),t9016(1)				CC5	ST225(21)	t003(21)
									CC5	(ST5)(4)	t010(2),t179(2)
									CC5	ST5(15)	t067(15)
									n.d.	n.d.(9)	t1062(1),t2173(1),t242(1),t264(1),t463(1),t777(3),t837(1)
2	5a(20)	7	CC5	ST5(14)	t002(14)	2	8(54)	18	CC8	ST8(35)	t008(35)
			CC5	(ST5)(4)	t010(1),t311(3)				CC8	ST239(9)	t030(6),t037(3)
			n.d.	n.d.(2)	t8921(2)				CC8	(ST8)(2)	t190(2)
									n.d.	n.d.(8)	t068(1)，t121(2),t1882(1),t2054(2),t2942(1),t801(1)

3	621(6)	2	singleton	ST2147(6)	t437(6)	3	22(39)	13	CC22	ST22(27)	t032(23),t515(3),t022(1)
									CC22	ST22(3)	t747(3)
									n.d.	n.d.(9)	t020(1),t025(1),t1214(1),t1865(1),t2231(1),t2357(1), t2945(1),t578(1),t885(1)
4	1(3)	1	CC1	ST401(2)	t127(2)	4	2(28)	11	CC5	ST228(7)	t001(7)
			singleton	ST2147(1)	t437(1)				CC5	ST111,ST228(18)	t041(18)
									n.d.	n.d.(3)	t1541(1),t1628(1),t2406(1)
5	398(3)	1	CC398	ST398(3)	t034(3)	5	45(17)	8	CC45	ST45(12)	t740(5),t038(3),t015(3),t004(1)
									n.d.	n.d.(5)	t1574(1)，t2418(1),t247(1),t2600(1),t630(1)

n.d.; not determined; n.t.; not typeable

Chapter 7

Genetic Features of Porcine *Staphylococcus aureus* Isolates from China that Carry the *lsa(E)* Gene for Quinupristin/dalfopristin Resistance

Xiaomei Yan[*], Zongwei Li[*], Monika A. Chlebowicz, Xiaoxia Tao, Ming Ni, Yuan Hu, Zhen Li, Hajo Grundmann, Xiaochen Bo, Jan Maarten van Dijl, Pengcheng Du, Minli Zhang, Yuanhai You, Xiaojie Yu, Fanliang Meng, Shengqi Wang[#], and Jianzhong Zhang[#]

* These authors contributed equally to this work

Abstract

The objective of this study was to investigate the genetic features of the newly identified *lsa(E)* gene that encodes an ABC transporter that mediates resistance to streptogramin A antibiotics in porcine *S. aureus* ST9 isolates by whole-genome sequencing (WGS). Three quinupristin/dalfopristin-resistant isolates harboring the *lsa(E)* gene (two MRSA and one MSSA) were sequenced. Single nucleotide polymorphisms (SNPs) were defined by comparisons with 31 publicly available *S. aureus* genomes in the NCBI database. Phylogenetic analysis showed that the sequenced isolates belong to a distinct evolutionary cluster closely related to clonal complex 5 (CC5). Further analysis showed that all isolates were deficient in the recently described type IV Restriction-Modification system. A novel type V (5C2)-like SCC*mec* element was identified in the two MRSA isolates, which included a class C2 *mec* gene complex and a new allotype *ccrC* gene. A 24kb ΨSCC fragment was found both in the MRSA and MSSA isolates sharing 99% nucleotide sequence homology with the ΨSCCJCSC6690 (O-2) element of a ST9 MRSA isolate from Thailand (accession number AB705453). Altogether, our WGS analysis indicates that the sequenced quinupristin/dalfopristin-resistant ST9 lineage represents a reservoir of new mobile genetic elements associated with new resistance features that may have originated from other species and spread further to human epidemic *S. aureus* lineages.

Introduction

Staphylococcus aureus is a Gram-positive bacterium able to colonize humans and various animal species. This opportunistic pathogen has the ability to rapidly develop resistance to any antibiotic that is in current clinical use (1). Up to now, a high prevalence of methicillin-resistant *S. aureus* (MRSA) in hospitals has been reported in many countries world-wide including China (2,3).

Antibiotic resistance in *S. aureus* can emerge in different ways which include point mutations of genes coding for antibiotic targets or horizontal gene transfer of mobile genetic elements (MGEs), such as plasmids, transposons or insertion (IS) elements, occasionally originating from other bacterial species (4-10). It has been reported, that genetic transfer of genes coding for antibiotic resistance can occur between *enterococci* and *S. aureus* (9,10). Resistance to vancomycin in *S. aureus* is associated with the acquisition of *vanA* located on a Tn*1546*-containing plasmid from enterococci. Transfer of resistance from *enterococci* to *S. aureus* has been reported to occur also for other resistance determinants, such as the tetracycline resistance gene *tet(L)*, the trimethoprim resistance gene *dfrK* (11) or the multiresistance gene *cfr* (12). Recently, a novel gene encoding the ABC transporter *lsa(E)*, which confers resistance to streptogramin A, has been identified in *S. aureus* that seems to originate from *Enterococcus* sp., as well (13).

Quinupristin and dalfopristin (collectively termed QDA) belong to the streptogramin A and B antibiotic group. These structurally distinct cyclic peptide antibiotics act synergistically on the bacterial 50S ribosomal subunit thereby inhibiting protein synthesis (14). Virginiamycin belongs to the same antibiotic group as QDA and is widely used as an animal growth promoter in poultry, cattle and swine. In *S. aureus,* resistance to streptogramin B does not generate resistance to QDA while resistance to streptogramin A does (15). Even though QDA has not been marketed in China, virginiamycin is widely used. Resistance to streptogramin A-type antibiotics can be caused by different mechanisms that involve factors, such as the acetyltransferase Vat (16-18) the ABC transporters Vga (19-21) and Lsa (13), and the methyltransferase Cfr (22).

Our previous study showed that 98% (44/45) of the QDA-resistant *S. aureus* isolates sampled from slaughter pigs in northeastern China harbored *lsa(E)*, which was also described recently in other regions of China (23-25). So far, the gene *lsa(E)* has only been found in isolates belonging to the sequence type (ST) 9 (both MRSA and MSSA) in China. Whether a deficiency of DNA restriction-modification system in ST9 isolates facilitates the horizontal gene transfer of foreign DNA, as has been reported for ST398 (26) is still unknown.

The objective of this study was to investigate the genetic features of new *lsa*(E)-positive porcine *S. aureus* ST9 isolates by whole-genome sequencing (WGS).

Materials and methods

Bacterial Strains, growth conditions and genomic DNA isolation

Three QDA-resistant isolates harboring the *lsa(E)* gene were selected for genome sequencing. Two of these isolates were MRSA and one was a methicillin-sensitive *S. aureus* (MSSA; Table 1). All three isolates were collected from nasal swabs from healthy pigs in Harbin city, Heilongjiang Province, in northeastern China. All isolates belonged to ST9 and had the *spa* type t899. Isolates were grown on tryptic soy agar (TSA) with 5% sheep blood at 37°C. Genomic DNA of each isolate was prepared using the QIAamp DNA Mini Kit (QIAGEN 51306, GmbH, Hilden, Germany) according to the manufacturer's protocol with an additional cell lysis step involving incubation with 50µg/ml lysostaphin (final concentration) at 37°C for 1 h.

Table 1. Characteristics of three *lsa(E)* positive *S. aureus* isolates.

Isolate	Source	Sample	MLST	*spa*	*mecA*	*pvl*	MIC of	*lsa(E)*	Antibiotic resistance
A69	pig	nose	9(CC9)	t899	+	-	8	+	FOX-CHL-TET-GEN-CIP-
A71	pig	nose	9(CC9)	t899	+	-	8	+	FOX-CHL-TET-GEN-CIP-
A187	pig	nose	9(CC9)	t899	-	-	8	+	CHL-TET-GEN-CIP-ERY-CLI-

*FOX, cefoxitin; CIP, ciprofloxacin; ERY, erythromycin; TET, tetracycline; CLI, clindamycin; GEN, gentamicin; CTT, chlortetracycline; CHL, chloramphenicol; TRS, trimethoprim/sulfamethoxazole; QDA, quinupristin/dalfopristin;

Library preparation

Genomic DNA (gDNA) (1µg per sample) was fragmented with a Covaris S220 focused-ultrasonicator (Covaris, part # SE-501-1001) under the following conditions: duty factor (10%), peak incident power (175), cycles per burst (200), duration (40 seconds), mode frequency sweeping (frequency sweeping) and temperature (5.5° to 6°C). Libraries were prepared using the Truseq DNA sample preparation kit (FC-121-2001, Illumina) according to the TruSeq DNA sample preparation guide (Part # 15026486 Rev. C, Illumina).

Genome sequencing, assembly and annotation

Genome sequencing was performed using the Illumina MiSeq sequencing system (Illumina, San Diego, CA 92122 USA) according to the MiSeq system user guide. *De novo* assemblies were performed using SOAPdenovo v1.05 (Beijing Genomics Institute at Shenzhen,

Shenzhen 518083, China) at an optimal hash length of 107, 107 and 109 for the A69, A71 and A187 strains, respectively. The published genome sequence of *S. aureus* N315 (gi|29165615|ref|NC_002745) was used as the reference genome. Contigs of each strain were resorted according to the N315 sequence by MAUVE (27). All three genome assemblies in this paper have been deposited at DDBJ/EMBL/GenBank under the accession numbers JJOP00000000, JJOO00000000 and JJON00000000. The version described here is the first version JJOP01000000, JJOO01000000 and JJON01000000 (to submit the results to NCBI, scaffolds that contain more than 10 continuous Ns were splitted into smaller contigs and the contigs less than 200bp were filtered out). Pairwise genomic comparisons were generated by blast and analyzed using the Artemis Comparison Tool (ACT) (http://www.sanger.ac.uk/resources/software/artemis/) (28).

Generated contigs where annotated using the Rapid Annotations Subsystems Technology (RAST) (29) and Artemis. tRNA- and rRNA-encoding genes were searched by tRNAscan-SE (30) and RNAmmer, separately (31). Gaps were amplified by PCR using Takara LA Taq polymerase, and the resulting PCR products were sequenced by primer walking (Table S1).

Phylogenetic analysis

The MUMmer 3.23 (32) and Trf (33) algorithms were used for identification of SNPs by aligning contigs of each strain to the reference genome of 31 published *S. aureus* genomes in the NCBI database. The MUMmer results of each strain were filtered to remove SNPs which might be unreliable according to the following criteria: 1) quality scores < 20 (average base calling error rate>0.01); 2) covered by < 10 paired-end reads; 3) in repetitive regions (Trf) (34). Based on the obtained core genome SNPs, the phylogenetic tree was generated using Phylip (http://evolution.gs.washington.edu/phylip.html) and MEGA (35). MRSA252 was chosen as the outgroup in the phylogenetic tree.

Isolation of plasmid DNA

Plasmid DNA of the three isolates was extracted using the Qiagen plasmid extraction midi kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with the following modification: after re-suspending the bacterial pellet in buffer P1, lysostaphin was added to a final concentration of 0.02mg/L and the mixture was incubated for 1 h at 37ºC before adding buffer P2. To estimate plasmid sizes, digestion using the restriction enzymes *Hin*dIII and *Eco*RI was performed separately on each isolated plasmid.

Results

Genomic characteristics of the sequenced ST9 isolates. In this study, two MRSA isolates (A69, A71) and one MSSA isolate (A187), have been analysed by whole genome sequencing. The circular chromosomes of isolates A69, A71 and A187 were shown to consist of 2,844,421 bp, 2,849,873 bp and 2,786,770 bp, respectively. Each isolate contains a single relatively small plasmid that slightly differs in size between isolates (3,027 bp, 2,581 bp and 2,990 bp, respectively, Table 2).

Table 2. General properties of the genomes of the *S. aureus* ST9 isolates A69, A71 and A187 and *S. aureus* N315.

Element and characteristic	A69	A71	A187	N315[45]
Chromosome				
Size (bp)	2,844,421	2,849,873	2,786,770	2,813,641
G+C content (%)	32.80%	32.80%	32.80%	32.80%
No. of coding sequences	2779	2781	2671	2595
tRNA genes	55	59	54	62
Transposons				
Tn552	1	1	1	0
Tn554	0	0	0	5
Tn558	1	1	1	0
Others	2	2	2	0
Bacteriophages	1	1	0	1
SCC*mec*	1	1	0	1
Pathogenicity islands	2	2	1	3
Genomic islands	2	2	2	0
Plasmids				
Size (bp)	3,027	2,581	2,990	24,653
G+C content (%)	28.6	28.2	29.03	28.7
No. of coding sequences	2	2	2	29

Genomic comparisons of the MRSA isolates with the MSSA isolate revealed that the MSSA isolate lacks a prophage (ΦSa2int), a pathogenicity island (SaPI6∆a), and part of SCC*mec* (Table 2). Furthermore, the chromosomes of isolates A69, A71 and A187 show approximately 92.66%, 92.67% and 92.49% sequence identity with that of *S. aureus* N315. The major differences observed concern the presence or absence of mobile genetic elements (MGEs). MGEs identified in all three isolates are: the genomic islands α and β, encoding enterotoxins; SaPIbov4-like pathogenicity islands (*vwb, scn, aadE*); the

transposons Tn552 (*blaZ*) , Tn552-like (*blaZ*), Tn558 (*fexA*); a transposon-like MGE carrying the *lsaE, lun(B), aadE,* and *tetL* genes; and an IS element (*dfrG*). The transposon Tn552 found in isolate A71 carried a truncated *blaZ* gene (Table 3).

Table 3. Summary of the major mobile genetic elements (MGEs) present in the three sequenced *S. aureus* ST9 isolates associated with virulence and antibiotic resistance.

	A69	A71	A187
Bacteriophage			
ØSa2	NI	NI	-
Genomic islands*			
γSaα	*set(11),lpl(7)*	*set(11),lpl(7)*	*set(11),lpl(6)*
γSaß	entertoxin(7), *lpl*(3)	entertoxin(8), *lpl*(3)	entertoxin(8), *lpl*(3)
Pathogenicity islands			
SaPIbov4-like	*vwb, scn,aadE*	*vwb, scn,aadE*	*vwb, scn,aadE*
SaPI6∆a	NI	NI	-
SCC*mec*	*mecA*	*mecA*	
Plasmid	NI	NI	NI
Transposons			
Tn552	*blaZ*	*blaZ(truncated)*	*blaZ*
Tn552-like transposon	*blaZ*	*blaZ*	*blaZ*
Tn558	*fexA*	*fexA*	*fexA*
Transposon-like MGE with	*lsaE,lun(B),aadE,tetL*	*lsaE,lun(B),aadE,tetL*	*lsaE,lun(B),aadE,tetL*
IS element	*dfrG*	*dfrG*	*dfrG*

NI, The MGE is present, but no virulence and antibiotic resistance gene was found.

*, the number of the virulence genes is indicated in parentheses.

New transposon carrying lsa(E)

Identical *lsa*(E) gene clusters were observed in all three genomes. The *lsa*(E) gene was embedded in a 12.2 kb MGE flanked by two IS257 sequences. Comparative analysis revealed that the *lsa*(E) gene cluster shows 99% nucleotide sequence identity with genetic structures (accession number JQ861959) found in one MRSA isolate that belongs to ST398 and two human MSSA isolates with ST9 from Spain. Furthermore, the identified MGE with the *lsa*(E) gene cluster shows similarity to plasmid pEF418 of *E. faecalis* (accession number AF408195), plasmid pXD4 from *E. faecium* (accession number KF421157) and plasmid pV7037 from swine MRSA originating from China (accession number JX560992) (Figure 1).

The MGE carrying *lsa*(E) is positioned adjacent to another transposon with IS257 containing the *tet*(L) tetracycline resistance gene upstream, which is 99% identical to a gene cluster found on *B. cereus* plasmid pBC16 (accession number AAA84922), the *E. faecium* plasmid pM7M2 (accession number JF800907), and the *S. aureus* SA7037 plasmid pV7037 (accession number HF586889).

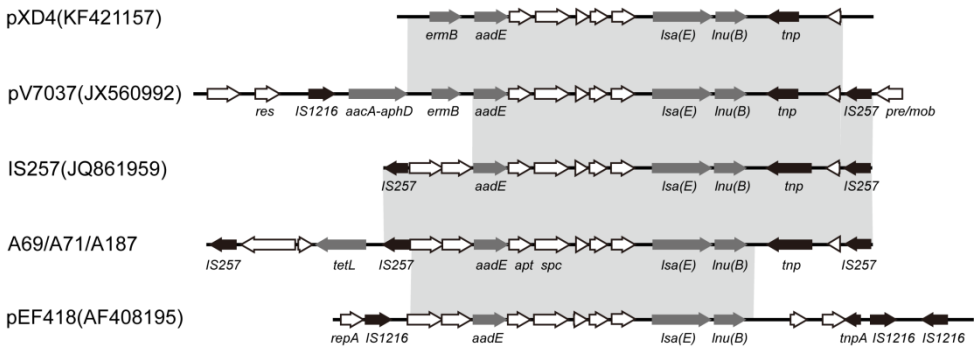

Figure 1. Structure of the genetic environment of the *lsa*(E) gene of *S. aureus* isolates A69/A71/A187 and comparison with homologous structures. The genetic environment of the lsaE genes of the *S. aureus* isolates A69, A71 and A187 were compared to homologous regions of the *S. aureus* plasmid V7037(XJ560992), the *S. aureus* transposon *IS*257 (JQ861959), the *E. faecalis* plasmid pEF418(AF408195) and the *E. faecium* plasmid pXD4(KF421157). The arrows represent the positions and orientations of the genes. Similar regions in the different structures are indicated by grey shading.

A new SCCmec structure identified in sequenced ST9 MRSA isolates

The SCC*mec* region of the sequenced MRSA isolates was carefully analyzed and a novel structure of this mobile element associated with methicillin resistance was observed (Figure 2). The newly identified SCC*mec* is 48.575 kb in length and contains 45 predicted open reading frames (Table S2). The G+C content of this SCC*mec* is 31.98%, which is slightly lower than the overall G+C content of 32.8% of the remaining chromosome. Furthermore, the SCC*mec* contains three integration site sequences (ISS) consisting of direct repeats (DR)

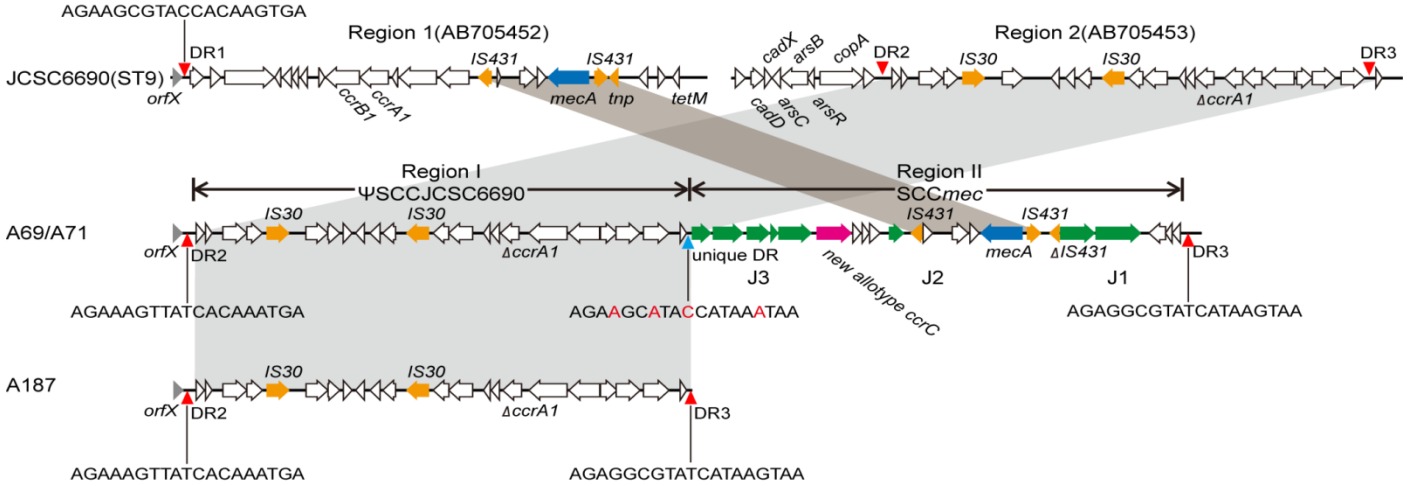

Figure 2. Structure of the type V (5C2)-like SCC*mec* and pseudo (Ψ)SCC elements of the sequenced MRSA isolates (A69/A71) and homologous regions in the MSSA isolate A187 and *S. aureus* JCSC6690 (O2) from Thailand. The SCC*mec* structures are illustrated based on the nucleotide sequences deposited in the GenBank database (AB705452 and AB705453). The red and blue arrowheads indicate the location of DR's with the respective sequences shown underneath. ORFs are indicated by arrows colored as follows: orange, insertion sequences; green, genes presumable acquired from other species; blue, *mecA*; pink, new allotype *ccrC*. The similarities in the different structures are indicated by grey shading.

that divide this MGE into two regions. The first DR is located within *orfX* and is identical to DR2 present in SCC*mec* JCSC6690 (O-2). The second DR, unique for this SCC*mec*, delimits region one and two, while the third DR is located at the end of this mobile element and is identical to DR3 on SCC*mec* JCSC6690 (O-2) (Figure 2). Region I, downstream of *orfX*, is 24.293kb in size and contains a pseudoSCC element with a truncated *ccrA1* gene. Overall the structure of this region shares 99% of nucleotide sequence identity with ΨSCCJCSC6690 (O-2) located on the SCC*mec* element of ST9 MRSA strain JCSC6690 (accession number AB705453). Region II is 24.282 kb in size and contains the *ccrC* gene cluster and a *mec* gene complex. A new allotype of *ccrC* was found with 68%-70% DNA sequence similarity with known staphylococcal *ccrC1* alleles (Table S3). This new allotype of *ccrC* shows 98% amino acid identity with a phage transcriptional activator of *S. epidermidis*. Additional hypothetical genes in the neighbourhood of *ccrC* are highly similar to proteins from *S. hominis*. Further, within the described region a 13.8 kb typical class C2 *mec* gene complex is located, after which a restriction modification-like structure is present. This new restriction modification-like structure consists of two novel proteins that share low overall similarity (45% and 31%) in amino acid sequence with a known methyltransferase (accession number WP_005807159.1) and a type III restriction enzyme (accession number WP_000891153.1), respectively. The newly identified SCC*mec* region does not contain any additional antimicrobial resistance determinants.

Interestingly, a remnant of the identified SCC*mec* element, including the DR2 and DR3 direct repeats, is present on the genome of the MSSA isolate. This SCC*mec* remnant of 24.7 kb comprises 23 predicted genes that are identical to equivalent genes present within the respective regions of the SCC*mec* elements of both sequenced MRSA isolates (Figure 2).

Phage and Pathogenicity Islands

Only one prophage Sa2*int* was identified in both MRSA genomes, which is similar to the prophage previously found in the sequenced *S. aureus* ST398 genome (SO385 strain). The present prophage Sa2*int* of 44 kb is integrated within the gene coding for 6-phospho-beta-galactosidase (*lacG*). This phage does carry any known virulence determinants (Table 3).

All three sequenced ST9 isolates carry a SaPIbov4-like pathogencity island, integrated downstream of the *guaA* gene for GMP synthase. In general, this mobile element is very similar in structure to the previously reported SaPIbov4 (accession number HM211303.1). However, clear differences are detectable at the 3' terminal part. The identified SaPI carries

the genes for an animal-associated staphylococcal complement inhibitor (*scn*) and a von Willebrand factor binding protein (*vwb*) that both have an inverted orientation compared to SaPIbov4 (Figure S1). Additionally, this new SaPIbov4-like element encodes an aminoglycoside 6-adenylyltransferase (*aadE*) in the 3' terminal part.

MGEs from other species

Except for the *lsa*(E) gene cluster and the novel SCC*mec* structure, two transposon-like elements were found in all three sequenced genomes. The first transposon-like element is inserted into the gene for the L-lactate permease (accession number EHM74991.1). It carries six genes including the gene for a Tn552 transposase (Table 4). This novel gene structure is physically linked with a Tn552 transposon carrying *blaZ.* The second transposon-like element is inserted downstream the gene for a hypothetical protein belonging to an enterotoxin homology group (accession number WP_001792564.1), and it carries four genes not found in *S. aureus* before (Table 4).

Phylogenetic relationships of the sequenced ST9 isolates with other sequenced *S. aureus* isolates

To determine the phylogenetic relationships between the A69, A71 and A187 isolates and other *S. aureus* types, their genome sequences were compared with 31 *S. aureus* genomes publicly available on NCBI that represent all major clonal lineages of *S. aureus*. When all 34 genomes were compared to each other, 125,578 SNPs were identified among which 152 SNPs were different among the three ST9 isolates. These SNPs where then used to reconstruct a phylogenetic tree. The phylogenetic tree topology revealed that the ST9 isolates belong to a distinct clade (Figure 3). The genetic distance between the MSSA and MRSA isolates is small, but the MRSA isolates are more closely related. Moreover, the ST9 clade appears to have emerged from clonal complex 5 (CC5), but it seems to be distantly related to other livestock-associated lineages, such as ST398, ST151, ST133 and ST425.

Restriction modification system (RM) of ST9

To better understand the mechanisms of *lsa*(E) gene transfer, the RM systems of the three sequenced ST9 isolates were analyzed. The intact type I RM system was found in all three strains sharing 100% similarity with one copy of the *hsdR* gene, which encodes the restriction subunit, and two different copies of *hsdMS*, which encode proteins for recognition and modification of specific sequences. *HsdR* and two copies of *hsdM* were conserved with only few mutations compared to published sequences of other *S. aureus* isolates. All three strains

Table 4. Transposon-like elements in the three sequenced *S. aureus* ST9 isolates and association with respective genes from other specices

transposon-like element	ORFs	Protein	NCBI accession number*	Species(or Genus)
1	1	ArsR family transcriptional regulator	WP_002486904.1	*Staphylococcus epidermidis*
	2	permease	WP_020008110.1	*Salinicoccus albus*
	3	methyltransferase	WP_009384754.1	*Staphylococcus massiliensis*
	4	Tn552 transposase	WP_026066869.1	*Staphylococcus intermedius*
	5	ATP-binding protein	WP_019168760.1	*Staphylococcus intermedius*
	6	hypothetical protein	WP_019168761.1	*Staphylococcus intermedius*
2	1	TnpA	AAX38177.1	*Enterococcus casseliflavus*
	2	NimC/NimA family	WP_004193041.1	*Klebsiella pneumoniae, Escherichia coli*, *Salmonella, Enterobacteriaceae*
	3	partial DNA polymerase	WP_002303392.1	*Enterococcus faecium*
	4	partial,methyltransferase	WP_029751903.1	*Streptococcus suis*

*, the representative NCBI accession numbers are listed in the table.

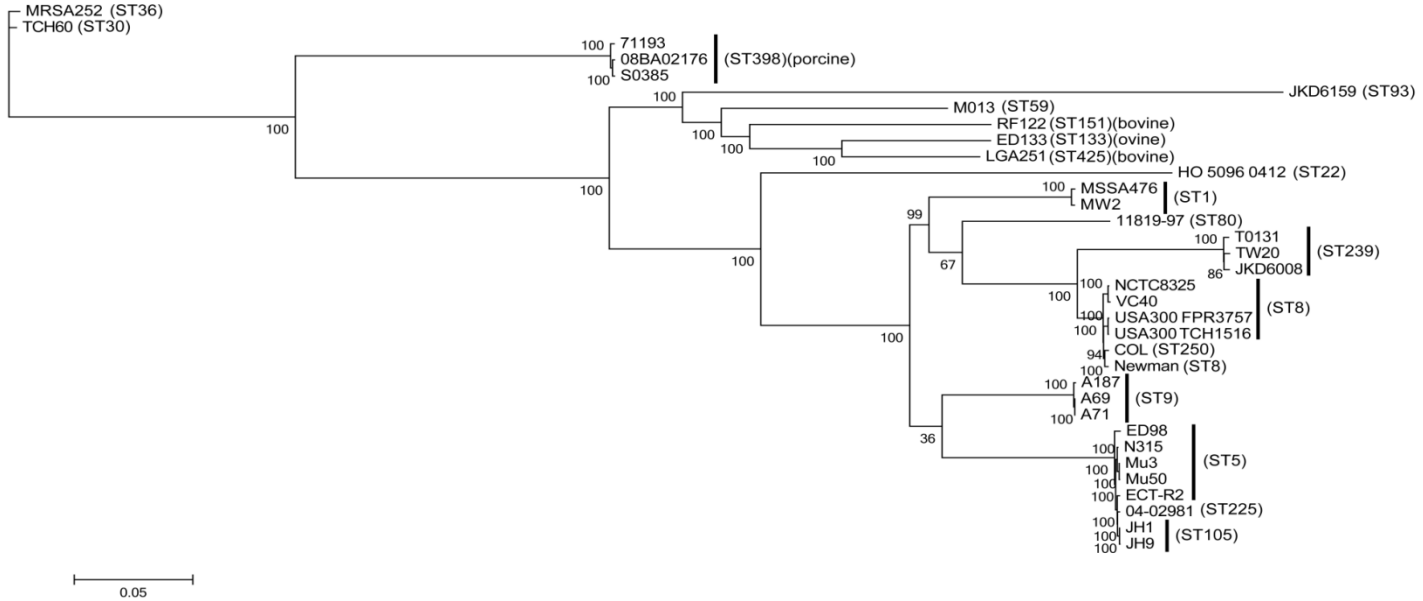

Figure 3. Phylogenetic relatedness of the ST9 clone with other major clonal lineages of *S. aureus*. The phylogenetic tree was generated based on core genome SNPs between the three sequenced ST9 isolates and 31 genomes of *S. aureus* belonging to other different ST's that were available on NCBI. MRSA252 was chosen as an out group for the phylogenetic tree. The ST of each strain is indicated. The scale indicates the genetic distance. The original habitat of livestock-associated isolates is indicated in parentheses.

possessed an amino acid substitution in the *hsdR* gene from arginine to lysine (R873K) when compared with *hsdR* of the N315 and 21334 strains (Figure S2). The genes for the methyltransferase HsdM and the specificity subunit HsdS, are located within the genomic islands vSaα and vSaβ, respectively. There is, 100% sequence similarity between *hsdM1* located on vSaα and the *hsdM* gene of strain 21334. The second copy of *hsdM2* located on vSaβ contains one amino acid substitution (S60A) compared to the *hsdM* gene of the 21334 strain (Figure S2). The lineage-specific *hsdS1* gene located within γSaα contains a specific region (encoding amino acid residues 24-171) that seems to be unique for HsdS1 as no similar sequence has been found in the NCBI database. On the other hand, *hsdS2* located in γSaβ encodes a protein with 99% amino acid sequence similarity to the HsdS2 proteins of strains 21334, MO483, Co-08 and KT/Y21 (Figure S2). Furthermore, genes for the intact type II RM-*Sau*3AI were found on the chromosomes of the sequenced ST9 isolates. This RM system consists of two enzymes, a restriction enzyme and the cognate modification enzyme. Interestingly, all isolates also possess a recently described type IV RM system (originally referred to as a type III system) that appears defective due to the loss of the corresponding *hsdR* gene as confirmed by PCR. Moreover, the rest 41 *lsa(E)* carrying strains were all deficient of *hsdR* according to PCR detection.

Discussion

This is the first study to describe the genetic features of *S. aureus* carrying the *lsa(E)* gene for quinupristin/dalfopristin resistance. Although ST9 (CC9) currently seems to be a dominant clone among slaughter pigs (24) in China, there appears to be no evolutionary link between this lineage and the lineage ST398 which is successfully spreading among pigs in Europe. Clearly ST9 emerged from a different evolutionary background than other typical livestock-associated CCs of *S. aureus*. Our phylogenetic analysis based on WGS revealed that the ST9 lineage most probably evolved from a recent common ancestor shared with *S. aureus* CC5. In fact, CC5 is one of the most successful healthcare-associated lineages within which the emergence of vancomycin resistant *S. aureus* (VRSA) has been reported in the USA (36). Furthermore, a subtype of CC5 was shown to be involved in a host jump from humans to poultry in Poland (37). Importantly, it was reported that ST9-MSSA can colonize humans and that it may have been transmitted between humans and pigs in the United Kingdom (38). It would therefore be of interest to explore whether host jumps between humans and pigs could be responsible for the emergence of ST9 as a livestock-associated *S. aureus* lineage.

Intriguingly, the investigated ST9 isolates are deficient in the type IV RM system, which

potentially facilitates the transfer of foreign DNA. Novel acquired DNA may thus convey novel phenotypic properties, such as the observed QDA resistance. This view is underpinned by the recent observation that a deficiency in the type IV RM system made *S. aureus* cells prone to the acquisition of the *vanA* gene from enterococci (39). It is thus conceivable that the deficiency of this particular type IV RM system has played a role in the genetic transfer of *lsa(E)* from enterococci to *S. aureus.* Additionally, two novel transposon-like elements were found in the investigated isolates. The respective genes showed a high degree of similarity to genes from coagulase negative staphyloccci or enterococci, and have never before been reported for *S. aureus*. This would support the hypothesis that a nonfunctional RM system could enhance the uptake of foreign DNA by isolates of the ST9 lineage. Further research needs to be performed to verify this hypothesis.

According to the nomenclature criteria for SCC*mec* types (IWG-SCC, 2009), the SCC*mec* elements in the two presently sequenced MRSA isolates belong to the type V (5C2)-like SCC*mec* elements with a class C2 *mec* gene complex and a new allotype *ccrC* gene complex. Except for the *mec* gene complex, the overall structure of this SCC*mec* is so far unique. The encoded genes are similar to genes from coagulase-negative staphylococci, indicating a possible origin for this SCC*mec* element. A relatively high frequency of *ccrC positive SCCmec was found in S. hominis, which suggest that S. hominis* could serve as a reservoir of this type of SCC*mec* for *S. aureus* (40). A pseudo (Ψ) SCC element with a truncated *ccrA* gene located in the left extremity of the SCC*mec* identified in this study showed high similarity to a ΨSCC element found in type IX SCC*mec* of a ST9 MRSA isolate from Thailand (41).

The novel SCC*mec* structure identified in the present study indicates that multiple recombination events have occurred in this region of the *S. aureus* genome, as has also been observed for other SCC*mec* types (42). Surprisingly, the intact ΨSCC element was also found in the presently sequenced MSSA isolate. Combined with our phylogenetic analyses, this suggests that the SCC*mec* remnant in the MSSA strain may have been derived from a MRSA ancestor that lost the mobile region containing the *mec* and *ccr* gene complexes. Such a partial loss of SCC*mec* has previously been observed to occur during a human infection (43). It has also been reported that under specific circumstances, such as the absence of selective antibiotic pressure, SCC*mec* may be unstable. Its excision from the chromosome would then result in a MSSA strain that could still carry a larger number of resistance determinants than usually found in native MSSA isolates (44). The MSSA isolate investigated in the present study was multiply resistant and carried the same resistance genes as both

investigated MRSA isolates except the *mecA* gene for methicillin resistance. Whether the high resistance of the MSSA isolate can be linked to a selective pressure in the animal feeding chain needs to be further investigated.

In summary, the ST9 lineage of *S. aureus* seems to be phylogenetically related to the *S. aureus* CC5, but not other livestock-associated lineages, such as ST398. Furthermore, we observed that the three sequenced ST9 isolates are deficient in the type IV RM system. These genetic features suggest that the ST9 lineage of *S. aureus* may serve as a reservoir of potentially new MGEs, such as SCC*mec,* SaPI and transposons originating from other species.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant No. 81301463), by the State Key Laboratory for Infectious Disease Prevention and Control Fund of China (2012SKLID202), and by an educational grant from the Graduate School of Medical Sciences of the University of Groningen, the Netherlands.

Supplementary Materials

Supplementary Table 1. Primers used in this study.

Supplementary Table 2. Genes of the SCC*mec* element in the two sequenced MRSA isolates.

Supplementary Table 3. Comparision of *ccrC* genes in this study with all known Staphylococcus *ccrC* alleles.

Supplementary Figure 1. Comparison of SaPIbov4 and the similar SaPI of the sequenced isolates A69/A71/A187.

Supplementary Figure 2. Amino acid sequence alignments of homologous proteins belonging to type I restriction modification systems.

Reference List

1. Pantosti, A., A. Sanchini, and M. Monaco. 2007. Mechanisms of antibiotic resistance in *Staphylococcus aureus*. Future. Microbiol. 2: 323-34.
2. Xiao, Y. H., C. G. Giske, Z. Q. Wei, P. Shen, A. Heddini, and L. J. Li. 2011. Epidemiology and characteristics of antimicrobial resistance in China. Drug Resist. Updat. 14: 236-50.
3. Edelsberg, J., D. Weycker, R. Barron, X. Li, H. Wu, G. Oster, S. Badre, W. J. Langeberg, and D. J. Weber. 2014. Prevalence of antibiotic resistance in US hospitals. Diagn. Microbiol. Infect. Dis. 78: 255-62.
4. Ferrero, L., B. Cameron, and J. Crouzet. 1995. Analysis of *gyrA* and *grlA* mutations in stepwise-selected ciprofloxacin-resistant mutants of *Staphylococcus aureus.* Antimicrob. Agents Chemother. 39:1554-8.
5. Howden, B. P., J. K. Davies, P. D. Johnson, T. P. Stinear, and M. L. Grayson. 2010. Reduced vancomycin susceptibility in *Staphylococcus aureus*, including vancomycin-intermediate and heterogeneous vancomycin-intermediate strains: resistance mechanisms, laboratory detection, and clinical implications. Clin. Microbiol. Rev. 23: 99-139.
6. Aubry-Damon, H., C. J. Soussy, and P. Courvalin. 1998. Characterization of mutations in the *rpoB* gene that confer rifampin resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. 42: 2590-4.
7. Turnidge, J. and P. Collignon. 1999. Resistance to fusidic acid. Int. J. Antimicrob. Agents. 12 Suppl 2:S35-44.
8. Tsiodras, S., H. S. Gold, G. Sakoulas, G. M. Eliopoulos, C. Wennersten, L. Venkataraman, R. C. Moellering, and M. J. Ferraro. 2001. Linezolid resistance in a clinical isolate of *Staphylococcus aureus.* Lancet. 358: 207-8.
9. Weigel, L. M., D. B. Clewell, S. R. Gill, N. C. Clark, L. K. McDougal, S. E. Flannagan, J. F. Kolonay, J. Shetty, G. E. Killgore, and F. C. Tenover. 2003. Genetic analysis of a high-level vancomycin-resistant isolate of *Staphylococcus aureus.* Science. 302:1569-71.
10. Weigel, L. M., R. M. Donlan, D. H. Shin, B. Jensen, N. C. Clark, L. K. McDougal, W. Zhu, K. A. Musser, J. Thompson, D. Kohlerschmidt, N. Dumas, R. J. Limberger, and J. B. Patel. 2007. High-level vancomycin-resistant *Staphylococcus aureus* isolates associated with a polymicrobial biofilm. Antimicrob. Agents Chemother. 51: 231-8.
11. Lopez, M., K. Kadlec, S. Schwarz, and C. Torres. 2012. First detection of the staphylococcal trimethoprim resistance gene *dfrK* and the *dfrK*-carrying transposon Tn559 in enterococci. Microb. Drug Resist. 18: 13-8.
12. Liu, Y., Y. Wang, C. Wu, Z. Shen, S. Schwarz, X. D. Du, L. Dai, W. Zhang, Q. Zhang, and J. Shen. 2012. First report of the multidrug resistance gene *cfr* in *Enterococcus faecalis* of animal origin. Antimicrob. Agents Chemother. 56: 1650-4.
13. Wendlandt, S., C. Lozano, K. Kadlec, E. Gomez-Sanz, M. Zarazaga, C. Torres, and S. Schwarz. 2013. The enterococcal ABC transporter gene *lsa(E)* confers combined resistance to lincosamides, pleuromutilins and streptogramin A antibiotics in methicillin-susceptible and methicillin-resistant *Staphylococcus aureus.* J. Antimicrob. Chemother. 68: 473-5.

14. Johnston, N. J., T. A. Mukhtar, and G. D. Wright. 2002. Streptogramin antibiotics: mode of action and resistance. Curr. Drug Targets. 3: 335-44.

15. Hancock, R. E. 2005. Mechanisms of action of newer antibiotics for Gram-positive pathogens. Lancet Infect. Dis. 5: 209-18.

16. Allignet, J., V. Loncle, C. Simenel, M. Delepierre, and S. N. el. 1993. Sequence of a staphylococcal gene, *vat*, encoding an acetyltransferase inactivating the A-type compounds of virginiamycin-like antibiotics. Gene 130: 91-8.

17. Allignet, J. and S. N. el. 1995. Diversity among the gram-positive acetyltransferases inactivating streptogramin A and structurally related compounds and characterization of a new staphylococcal determinant, *vatB.* Antimicrob. Agents Chemother. 39: 2027-36.

18. Allignet, J., N. Liassine, and S. N. el. 1998. Characterization of a staphylococcal plasmid related to pUB110 and carrying two novel genes, *vatC* and *vgbB*, encoding resistance to streptogramins A and B and similar antibiotics. Antimicrob. Agents Chemother. 42: 1794-8.

19. Allignet, J., V. Loncle, and S. N. el. 1992. Sequence of a staphylococcal plasmid gene, *vga*, encoding a putative ATP-binding protein involved in resistance to virginiamycin A-like antibiotics. Gene 117: 45-51.

20. Kadlec, K. and S. Schwarz. 2009. Novel ABC transporter gene, *vga(C),* located on a multiresistance plasmid from a porcine methicillin-resistant *Staphylococcus aureus* ST398 strain. Antimicrob. Agents Chemother. 53: 3589-91.

21. Schwendener, S. and V. Perreten. 2011. New transposon Tn6133 in methicillin-resistant *Staphylococcus aureus* ST398 contains *vga(E)*, a novel streptogramin A, pleuromutilin, and lincosamide resistance gene. Antimicrob. Agents Chemother. 55: 4900-4.

22. Long, K. S., J. Poehlsgaard, C. Kehrenberg, S. Schwarz, and B. Vester. 2006. The Cfr rRNA methyltransferase confers resistance to Phenicols, Lincosamides, Oxazolidinones, Pleuromutilins, and Streptogramin A antibiotics. Antimicrob. Agents Chemother. 50: 2500-5.

23. Li, X. S., W. C. Dong, X. M. Wang, G. Z. Hu, Y. B. Wang, B. Y. Cai, C. M. Wu, Y. Wang, and X. D. Du. 2014. Presence and genetic environment of pleuromutilin-lincosamide-streptogramin A resistance gene *lsa(E)* in enterococci of human and swine origin. J. Antimicrob. Chemother. 69: 1424-6.

24. Yan, X., X. Yu, X. Tao, J. Zhang, B. Zhang, R. Dong, C. Xue, H. Grundmann, and J. Zhang. 2014. *Staphylococcus aureus* ST398 from slaughter pigs in northeast China. Int. J. Med. Microbiol. 304: 379-83.

25. Li, B., S. Wendlandt, J. Yao, Y. Liu, Q. Zhang, Z. Shi, J. Wei, D. Shao, S. Schwarz, S. Wang, and Z. Ma. 2013. Detection and new genetic environment of the pleuromutilin-lincosamide-streptogramin A resistance gene *lsa(E)* in methicillin-resistant *Staphylococcus aureus* of swine origin. J. Antimicrob. Chemother. 68: 1251-5.

26. Schijffelen, M. J., C. H. Boel, J. A. van Strijp, and A. C. Fluit. 2010. Whole genome analysis of a livestock-associated methicillin-resistant *Staphylococcus aureus* ST398 isolate from a case of human endocarditis. BMC. Genomics 11: 376.

27. Darling, A. E., B. Mau, and N. T. Perna. 2010. Progressive Mauve: multiple genome

alignment with gene gain, loss and rearrangement. PLoS One. 5:e11147.

28. Carver, T. J., K. M. Rutherford, M. Berriman, M. A. Rajandream, B. G. Barrell, and J. Parkhill. 2005. ACT: the Artemis Comparison Tool. Bioinformatics. 21: 3422-3.

29. Aziz, R. K., D. Bartels, A. A. Best, M. DeJongh, T. Disz, R. A. Edwards, K. Formsma, S. Gerdes, E. M. Glass, M. Kubal, F. Meyer, G. J. Olsen, R. Olson, A. L. Osterman, R. A. Overbeek, L. K. McNeil, D. Paarmann, T. Paczian, B. Parrello, G. D. Pusch, C. Reich, R. Stevens, O. Vassieva, V. Vonstein, A. Wilke, and O. Zagnitko. 2008. The RAST Server: rapid annotations using subsystems technology. BMC. Genomics 9: 75.

30. Lagesen, K., P. Hallin, E. A. Rodland, H. H. Staerfeldt, T. Rognes, and D. W. Ussery. 2007. RNAmmer: consistent and rapid annotation of ribosomal RNA genes. Nucleic Acids Res. 35: 3100-8.

31. Schattner, P., A. N. Brooks, and T. M. Lowe. 2005. The tRNAscan-SE, snoscan and snoGPS web servers for the detection of tRNAs and snoRNAs. Nucleic Acids Res. 33: W686-9.

32. Kurtz, S., A. Phillippy, A. L. Delcher, M. Smoot, M. Shumway, C. Antonescu, and S. L. Salzberg. 2004. Versatile and open software for comparing large genomes. Genome Biol. 5: R12.

33. Benson, G. 1999. Tandem repeats finder: a program to analyze DNA sequences. Nucleic Acids Res. 27: 573-80.

34. Cui, Y., C. Yu, Y. Yan, D. Li, Y. Li, T. Jombart, L. A. Weinert, Z. Wang, Z. Guo, L. Xu, Y. Zhang, H. Zheng, N. Qin, X. Xiao, M. Wu, X. Wang, D. Zhou, Z. Qi, Z. Du, H. Wu, X. Yang, H. Cao, H. Wang, J. Wang, S. Yao, A. Rakin, Y. Li, D. Falush, F. Balloux, M. Achtman, Y. Song, J. Wang, and R. Yang. 2013. Historical variations in mutation rate in an epidemic pathogen, *Yersinia pestis.* Proc. Natl. Acad. Sci. U. S. A 110: 577-82.

35. Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28: 2731-9.

36. Limbago, B. M., A. J. Kallen, W. Zhu, P. Eggers, L. K. McDougal, and V. S. Albrecht. 2014. Report of the 13th vancomycin-resistant *Staphylococcus aureus* isolate from the United States. J. Clin. Microbiol. 52: 998-1002.

37. Lowder, B. V., C. M. Guinane, N. L. Ben Zakour, L. A. Weinert, A. Conway-Morris, R. A. Cartwright, A. J. Simpson, A. Rambaut, U. Nubel, and J. R. Fitzgerald. 2009. Recent human-to-poultry host jump, adaptation, and pandemic spread of *Staphylococcus aureus.* Proc. Natl. Acad. Sci. U. S. A. 106: 19545-50.

38. Armand-Lefevre, L., R. Ruimy, and A. Andremont. 2005. Clonal comparison of *Staphylococcus aureus* isolates from healthy pig farmers, human controls, and pigs. Emerg. Infect. Dis. 11: 711-4.

39. Corvaglia, A. R., P. Francois, D. Hernandez, K. Perron, P. Linder, and J. Schrenzel. 2010. A type III-like restriction endonuclease functions as a major barrier to horizontal gene transfer in clinical *Staphylococcus aureus* strains. Proc. Natl. Acad. Sci. U. S. A. 107: 11954-8.

40. Mendoza-Olazaran, S., R. Morfin-Otero, E. Rodriguez-Noriega, J. Llaca-Diaz, S.

Flores-Trevino, G. M. Gonzalez-Gonzalez, L. Villarreal-Trevino, and E. Garza-Gonzalez. 2013. Microbiological and molecular characterization of *Staphylococcus hominis* isolates from blood. PLoS One 8: e61161.

41. Lulitanond, A., T. Ito, S. Li, X. Han, X. X. Ma, C. Engchanil, A. Chanawong, C. Wilailuckana, N. Jiwakanon, and K. Hiramatsu. 2013. ST9 MRSA strains carrying a variant of type IX SCC*mec* identified in the Thai community. BMC. Infect. Dis. 13: 214.

42. Chlebowicz, M. A., T. Bosch, A. J. Sabat, J. P. Arends, H. Grundmann, J. M. van Dijl, and G. Buist. 2013. Distinction of Staphylococcal Cassette Chromosome *mec* type V elements from *Staphylococcus aureus* ST398. Int. J. Med. Microbiol. 303: 422-32.

43. Chlebowicz, M. A., K. Nganou, S. Kozytska, J. P. Arends, S. Engelmann, H. Grundmann, K. Ohlsen, J. M. van Dijl, and G. Buist. 2010. Recombination between *ccrC* genes in a type V (5C2&5) staphylococcal cassette chromosome *mec* (SCC*mec*) of *Staphylococcus aureus* ST398 leads to conversion from methicillin resistance to methicillin susceptibility *in vivo*. Antimicrob. Agents Chemother. 54: 783-91.

44. Donnio, P. Y., D. C. Oliveira, N. A. Faria, N. Wilhelm, C. A. Le, and L. H. de. 2005. Partial excision of the chromosomal cassette containing the methicillin resistance determinant results in methicillin-susceptible *Staphylococcus aureus*. J. Clin. Microbiol. 43: 4191-3.

45. Kuroda M, Ohta T, Uchiyama I, Baba T, Yuzawa H, Kobayashi I, Cui L, Oguchi A, Aoki K, Nagai Y, Lian J, Ito T, Kanamori M, Matsumaru H, Maruyama A, Murakami H, Hosoyama A, Mizutani-Ui Y, Takahashi NK, Sawano T, Inoue R, Kaito C, Sekimizu K, Hirakawa H, Kuhara S, Goto S, Yabuzaki J, Kanehisa M, Yamashita A, Oshima K, Furuya K, Yoshino C, Shiba T, Hattori M, Ogasawara N, Hayashi H, and Hiramatsu K. 2001. Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*. Lancet. 357: 1225–40.

Table S1. Primers used in this study

Primer	Sequence (5'→3')	Reference
Restriction system		
sau1hsdM1_F	TCAAAATTAGCTTGAAAGATGG	Lindsay, 2006
sau1hsdM1_R	AACCCTGGGAACCTCAATTCTGG	Lindsay, 2006
hsdM2_F2	GAATGTCTCAAATAAAGCCAATC	This study
hsdM2_R2	CCAAGTTCTTTCAGGTATGCAT	This study
hsdS1_F2	ATGAGTAATACACAAAAGAAAAATG	This study
hsdS1_R3	CAGATAAAACTTCTAGAAACCC	This study
sau1hsdS2_F2	TTAAATGAACAACTTTTGAAG	This study
sau1hsdS2_R2	ATGAGTAATACACAAAAGA	This study
type III_hsdR_F1	ATTGATCCCCAGAAAGCGCA	This study
type III_hsdR_R1	GCTGGATTCCATGCCAAAGG	This study
IS257 region		
IS257_L_F	CTGCGGTTCTTTTTATATAGAGCGT	This study
IS257_L_R	CACCAGCCTTCAGCATCCTA	This study
IS257_R_F	ATGACTACGGCAGAAATGAT	This study
IS257_R_R	GGTTCTGTTGCAAAGTTGA	This study
IS257_10_F	AGCCATTGCTACCTTCGTTG	Li B,2013
pre/mob_10_R	GACTGCTGCGAGAGATAACG	Li B,2013
	CTCATTCCAGCACCAGCTTCC	This study
	CCTAATACGATGCCTTCGGTT	This study
SCC*mec*		
orfX-F	AGGACGTCTTACAACGCAGT	This study
ccrA-R	AGCGCAGATTTTACGGTCTGA	This study
ccr-R-F	ACCATTGTTTAGAAGCAATCCCT	This study
mec-L-R	TCTTGTGTCAATTCACGTTCG	This study
c530-c544-F	GCAGCTCAATCTGTATGAGAC	This study
c530-c544-R	CATAGTTCCCATCTCGAATTTC	This study
c544-c530-F	TCATACGGCCCACACCAAAT	This study
s13-c530-R	CATATGGGTCTTCTCTTGTTGA	This study
mecA-F2	GTGATATGGAGGTGTAGAAGG	This study
t-RNA-R	AAGTTAGGTCGATCGCTTCTT	This study
A69_1-w2R	ACTTAAACCTGACTGTCATT	This study
A69_1-w1F1	TTAAACCTGACTGTCATTGT	This study
A69_1-w2R	ACTTAAACCTGACTGTCATT	This study
Phage and SaPI		
C522-F	CCCTCAGATGTTTTCAACCAC	This study

	S2-R	ACGCCTCTACGATGTTGGC	This study
	s1-c522-F	TGCTGCACGAACATCTTCAGG	This study
	s1-c522-R	GGTGTCATAAGGTTAGTACTG	This study
	s1-F	GTTGCACACCTTCCATCGTTTC	This study
	s2-R	CGGTATTAACGGGATTGATTGG	This study
	28-W1f	TGATTCTTTTCGCAACCTAT	This study
	28-w2f	CGTTGAGCAAAAACATTAGA	This study
	28-w1r	CGGTGGATGGACTGACGGAA	This study
	28-w2r	AGCATACATGGATGGTAAAA	This study
	s2-F	GATCTTCAATCGCGCGTTTGT	This study
	s14-R	GAAGAGGATACAAACCCAGGT	This study
Transposon			
	s6-F	CAACCGTTGAAGGAGACCAG	This study
	s25-R	GGCGCATATTAGTTGTAACTGG	This study

Table S2. Genes of the SCC*mec* element in the two sequenced MRSA isolates

ORFs	start	end	strand	gene name	Description
1	1115	1507	sense		hypothetical protein
2	1563	1880	sense		glycerol-3-phosphate permease-like protein
3	2395	3546	sense		hypothetical protein
4	3542	4294	sense		CAAX amino terminal protease family
5	4488	5588	sense	*tnp*	IS30 family transposase
6	6376	7425	sense		L-lactate permease
7	7426	7998	sense		hypothetical protein
8	8160	8585	sense		hypothetical protein
9	8749	9171	antisense		ISCpe6, transposase orfB
10	9439	9924	antisense		tranposase-like
11	10046	10666	antisense		regulatory protein GntR, HTH
12	11166	12266	antisense	*tnp*	IS30 family transposase
13	12460	13212	antisense		CAAX amino terminal protease family
14	13208	14359	antisense		hypothetical protein
15	14874	15191	antisense		glycerol-3-phosphate permease-like protein
16	15247	15639	antisense		hypothetical protein
17	15692	16579	antisense	truncated *ccrA1*	truncated cassette chromosome recombinase A1
18	17049	18881	antisense		hypothetical protein

19	18965	20437	antisense		hypothetical protein
20	20479	21273	sense		hypothetical protein
21	21224	22378	sense		hypothetical protein
22	22537	23763	sense		hypothetical protein
23	24295	24603	sense		acetyltransferase
24	24852	25805	sense		hypothetical protein (67% identity with *S.hominis*)
25	25853	27337	sense		hypothetical protein (95% identity with *S.hominis*)
26	27479	28615	sense		hypothetical protein (90% identity with *S.hominis*)
27	28620	29006	sense		hypothetical protein (99% identity with *S.hominis*)
28	28999	30612	sense		primase(93% identity with *S.hominis*)
29	30833	32524	sense	new	cassette chromosome recombinase C
30	32562	32951	sense		hypothetical protein
31	33017	33355	sense		hypothetical protein
32	33365	33874	sense		hypothetical protein
33	34291	35037	sense		hypothetical protein (93% identity with *S.hominis*)
34	35318	36010	antisense	*tnp*	IS431 transposase
35	35930	36397	sense		hypothetical protein
36	37326	38096	sense		glycerophophory diester phosphodiesterase

37	38178	38621	sense		MaoC domain protein dehydratase
38	38667	40694	antisense	*mecA*	penicillin-binding protein 2
39	40903	41598	sense	*tnp*	IS431 transposase
40	41963	42472	antisense		truncated IS432 transposase
41	42490	44178	sense		methyltransferase (45% identity with *Bacteroides fragilis*)
42	44185	46380	sense		hypothetical protein (29% identity with *Bacillus cereus*)
43	46746	47546	antisense		hypothetical protein
44	47540	47860	antisense		hypothetical protein
45	47861	48250	antisense		hypothetical protein

Table S3. Comparision of *ccrC* genes in this study with all known Staphylococcus *ccrC* alleles

strains	species	accession no.	sizes (bp)	allotypes and alleles	% identity to allele 1	allele 2	allele 3	allele 4	allele 5	allele 6	allele 7	allele 8	allele 9
WIS	*S. aureus*	AB121219	1623	C1 allele 1	100	91.4	89.9	88.4	92.1	91.6	89.1	89.7	91.4
TSGH17	*S. aureus*	AY894416	1680	C1 allele 2	91.4	100	96.1	88.6	95.6	97.4	93.9	92.5	87
85/2082	*S. aureus*	AB037671	1554	C1 allele 3	89.9	96.1	100	88.7	94.3	96.3	97.9	96.2	88.9
M	*S. aureus*	U10927	1683	C1 allele 4	88.4	88.6	88.7	100	90.3	89.1	90.1	90.9	88
JCSC1435	*S. haemolyticus*	AP006716	1677	C1 allele 5	92.1	95.6	94.3	90.3	100	96.4	93	92.1	87.6
25-60	*S. haemolyticus*	EF190467	1677	C1 allele 6	91.6	97.4	96.3	89.1	96.4	100	94.4	93	87.7
13-48	*S. epidermidis*	EF190468	1677	C1 allele 7	89.1	93.9	97.9	90.1	93	94.4	100	97.5	89.9
PM1	*S. aureus*	AB462393	1677	C1 allele 8	89.7	92.5	96.2	90.9	92.1	93	97.5	100	90.2
ATCC15305	*S. saprophyticus*	NC_007350	1683	C1 allele 9	91.4	87	88.9	88	87.6	87.7	89.9	90.2	100
A69	*S. aureus*	JJOP00000000	1686	new allotype	69.1	70.1	68	68.3	69.5	69.3	68.6	68.6	68.5

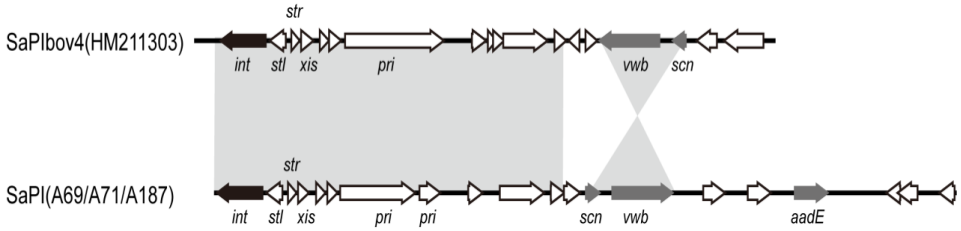

Figure S1. Comparison of SaPIbov4 and the similar SaPI of the sequenced isolates A69/A71/A187. The previously described structure of SaPIbov4 (HM211303.1) is shown on the top and the related SaPI from the A69/A71/A187 at the bottom. The arrows represent the positions and orientations of the different genes. Similar regions within the different structures are indicated by grey shading.

Figure S2. Amino acid sequence alignments of homologous proteins belonging to type I restriction modification systems. The alignments include inferred primary translation products of genes encoding the Sau1 restriction endonuclease subunit (HsdR), the modification subunit (HsdM) and the specificity subunit (HsdS). The *hsdM1* and *hsdS1* genes are encoded by the γSaα island, and the *hsdM2* and *hsdS2* genes by the γSaβ island. Homologous sequences of *S. aureus* N315, 21334, MSSA476, MO483, Co-08 and KT/Y21 and ED133 were derived from Genbank. Numbers above the sequences represent the amino acid positions in the prototype sequence shown in the first line of each alignment. Amino acid replacements are marked in green and blue. Identical sequences are marked in yellow.

Chapter 8

The Influence of SasX on the Epidemicity of *Staphylococcus aureus* ST 239 cannot be Explained by a Role in Colony Spreading Motility

Xiaomei Yan, Eleni Tsompanidou, and Jan Maarten Van Dijl

Abstract

Staphylococcus aureus can spread over wet surfaces by secreting phenol-soluble modulins with surfactant properties. This staphylococcal motility is antagonized by covalently cell wall-bound 'LPxTG' proteins, such as FnbpA, FnbpB, ClfA and ClfB. It has been proposed that spreading motility is a mechanism that contributes to host colonization by *S. aureus*. Recently, a novel covalently cell wall-bound protein, named SasX, was identified in hospital-acquired methicillin-resistant *S. aureus* (HA-MRSA) isolates from China of which many have the sequence type 239. This SasX protein was shown to promote inter-cellular aggregation of *S. aureus*. We therefore investigated, either by *sasX* deletion or *sasX* overexpression, whether the SasX protein may have an antagonizing influence on spreading motility. The results show that SasX by itself has no significant impact on colony spreading, suggesting that this protein's role in the epidemicity of *S. aureus* is unrelated to motility.

Introduction

Staphylococcus aureus is a major human pathogen that causes frequent and often severe infections throughout the world. Methicillin-resistant *S. aureus* (MRSA) isolates represent a major problem for public health due to their multiple antibiotic resistances. Recently, a novel surface protein named SasX was found in hospital-acquired MRSA (HA-MRSA) isolates from China. Most of these MRSA isolates belong to sequence type (ST) 239, which is the most predominant ST in China (11). SasX is a covalently cell wall-bound 'LPxTG protein', which is encoded by the 3' end region of a 127.2-kb ϕSPβ-like prophage. Interestingly, as a surface protein, SasX not only promotes adhesion of *S. aureus* to nasal epithelial cells and intercellular aggregation (e.g. increased biofilm formation), but it plays a key role in pathogenesis as well (11).

Bacterial motility plays an important role in the colonization of different environmental niches (6). *S. aureus* is able to move over wet surfaces by a mechanism that is known as colony spreading, which was first described in 2007 (8). Colony spreading of *S. aureus* is promoted by the secretion of so-called phenol-soluble modulins (PSMs) (18,20) and it is antagonized by the covalently cell wall-bound LPxTG proteins FnbpA, FnbpB, ClfA and ClfB (19). Accordingly, deletion of the four respective genes leads to a hyper-spreading phenotype (19). In addition, FnbpA and FnbpB have been shown to promote biofilm accumulation in certain HA-MRSA strains (13,21), while ClfA and ClfB have been implicated in cell-cell interactions (3,22). Noticeably, colony spreading motility and biofilm formation or cell aggregation are processes with opposite effects. Since SasX seems to have similar functions as FnbpAB and ClfAB, the objective of the present study was to investigate whether SasX also antagonizes colony spreading of *S. aureus*.

Material and methods

Bacterial Strains and Growth Conditions

The bacterial strains and plasmids that were used in this study are listed in Table 1. All *Escherichia coli* strains and *S. aureus* strains were, respectively, grown in lysogeny broth (LB) and tryptic soy broth (TSB) at 37°C under vigorous shaking. When necessary, ampicillin 100 µg/ml (for *E. coli*), erythromycin 5 µg/ml and chloramphenicol 12.5 µg/ml (for *S. aureus*) were added to the growth medium.

Colony spreading

The colony spreading assay was performed as described previously (20). Briefly, 10 ml TSB supplemented with 0.24% agar were added in each plate and dried for approximately 10 min in a laminar flow cabinet. From an overnight culture of *S. aureus* in TSB, an aliquot of 2 µl

was spotted in the center of a plate, which was subsequently dried for 10 seconds under laminar flow. The plates were then incubated overnight at 37°C. To induce *sasX* expression from the *sasX*-pCN51*cat* plasmid, 2.0 μM $CdSO_4$ was included in the soft agar plates. Images were recorded with a G: Box (Syngene, Leusden, Netherlands). The spreading area was analyzed with Image J software (1.47t). P-values were determined by the non-parametric Mann–Whitney U test.

Construction of a *sasX* mutant *S. aureus* strain

The *S. aureus* strain AH9, which harbors the *sasX* gene and has the ability to spread on soft agar plates, was used to construct a *sasX* deletion mutant with the help of the temperature-sensitive plasmid pMAD*cat* as previously described (1,17) (Fig. 1). All primers used are listed in Table 2. Briefly, DNA fragments of ～500-bp representing the upstream and downstream sequences of *sasX* were PCR-amplified from chromosomal DNA with the forward (F) and reverse (R) primer sets *sasX*-F1/RI and *sasX*-F2/R2. Then, a second PCR reaction was performed with the two fragments from the first reaction as templates, using primers F1/R2. The fused fragments were cloned into the chromosomal integration plasmid pMAD*cat,* the replication of which is impaired at 42^{o}C. The resulting plasmid *sasX*-pMADcat was first used to transform the *S. aureus* RN4220 strain, and then it was introduced into *S. aureus* AH9 via electroporation. Transformants were selected on plates with chloramphenicol and 80 ug/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. In order to delete *sasX* from the chromosome, cells with the chromosomally integrated *sasX*-pMADcat were selected by incubation at 42℃. Next, excision of *sasX*-pMADcat from the chromosome was triggered by incubation at 30 ^{o}C and white colonies that had lost the excised plasmid were screened for the absence of *sasX* by PCR with the primers *sasX*-F/R.

Overexpression of *sasX* in *S. aureus* **SH1000Δ**

To express *sasX* in *S. aureus* SH1000Δ, which lacks the *fnbpA*, *fnbpB*, *clfA* and *clfB* genes, the *sasX* gene was cloned in plasmid pCN51, which places it under the transcriptional control of a cadmium-inducible promoter. Since strain SH1000Δ is resistant to erythromycin, a chloramphenicol resistance gene (*cat*) was first incorporated into the pCN51 plasmid (Table 1). This *cat* gene was amplified from plasmid pRIT5H (7) with primers *cat*-F/R (Table 2) and ligated into pCN51 resulting in pCN51*cat.* Next, *sasX* was PCR-amplified with the primer set *sasX*-CN-F/R and ligated into plasmid pCN51*cat*, which resulted in plasmid *sasX*-pCN51*cat.* Both plasmids were used to transform *S. aureus* RN4220 cells, and then the SH1000Δ strain via electroporation.

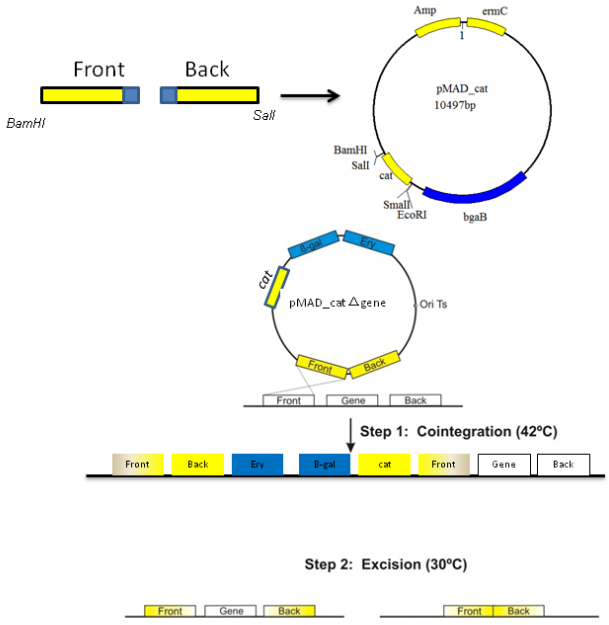

Figure 1. Schematic representation of the deletion of the *sasX* gene from *S. aureus* AH9. The flanking regions upstream and downstream of *sasX* are presented as yellow boxes marked “front” and “back”. These front and back regions were amplified and merged by PCR, and then cloned into the plasmid pMAD*cat.* Chromosomal integration of pMAD*cat* with the front and back regions (step 1) can occur by single cross-over recombination upstream or downstream of the *sasX* gene. A second recombination (step 2) can take place either at the front or back region. According to the place where the second recombination occurs, the *sasX* gene will either be excised (right), or remain intact on the chromosome (left).

Table 1 Bacterial strains and plasmids used in this study

Strains	**Genotype**	**Reference**
E. coli		
E. coli DH5α	λ⁻ φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA hsdR17*(r_k^- m_k^-)*sup*E44 *thi-1 gyrA relA1*	(5)
S. aureus		
RN4220	Restriction-deficient derivative of NCTC8325, cured of all known prophages	(9)
SH1000Δ	*ΔfnbpA, ΔfnbpB, ΔclfA, ΔclfB*::Em^R	(4)
SH1000ΔpCN51*cat*	SH1000Δ with plasmid pCN51*cat*[a];,Em^R, Cm^R	This work
SH1000Δ*sasX*	SH1000Δ with plasmid *sasX*-pCN51*cat*; Em^R, Cm^R	This work
AH9	clinical isolate; *sasX*-proficient	This work
AH9Δ*sasX*	Δ*sasX*	This work

Plasmids

Plasmids	**Relevant properties**	**Reference**
pMAD*cat*	pMAD carrying a chloramphenicol resistance gene	(17)
pCN51	E. coli/*S. aureus* shuttle vector that contains a cadmium-inducible promoter; Ap^R, Em^R	(2)
pCN51*cat*	pCN51 carrying a chloramphenicol resistance gene	This work
sasX-pCN51*cat*	pCN51*cat* with the *sasX* gene; Ap^R, Em^R, Cm^R	This work
pRIT5H	*E. coli/S. aureus* shuttle vector, Cm^R	(10)

Ap, Ampicillin; Em, Erythromycin; Cm, Chloramphenicol

[a], *S. aureus* doesn't become resistant to Ap by the presence of pCN51*cat*

Table 2. Primers used in this study

Primer	Sequence (5'→3')	**Reference**
For sasX detection		(11)
sasX-R	GCTGATTATGTAAATGACTCAAATG	
For sasX mutation		This work
sasX-F1	AAAAGGATCCAGTATCTCTACCTCTCTC	
sasX-R1	**CTACGTCAGTCAGTCACCATGGCA**GTATCATATCCATAAAACAC	
sasX-F2	**TGCCATGGTGACTGACTGACG**CACGGTTTAAATATTCAACCCG	
sasX-R2	CGAGTCGACGTTTAACAGGTGGCTTTCGAG	
For generating	AAAAGGATCCGATAAAGTGGGATATTTT	(17)
cat-R	CGAGTCGACCGGGGCAGGTTAGTGACATT	
For sasX expression	AAAAGGATCCGACAAGGACATTATATGTCCAC	This work
sasX-CN-R	AAAAGAATTCCCCCTTGATTAAGAAATCAAAG	

Overlapping nucleotides are shown in bold; restriction sites in primers are underlined; F, forward primer; R, reverse primer.

Results and Discussion

PCR screening for the presence of *sasX* was performed on 60 clinical MRSA isolates from China that belong to ST239. This resulted in the identification of 22 *sasX*-proficient isolates. Six *sasX*-proficient isolates were assayed for their colony spreading ability on soft agar plates. The results showed that four of these isolates displayed colony spreading motility.

To investigate whether SasX can have an influence on colony spreading, similar to FnbpA/B and ClfA/B, a s*asX* mutant of the spreading proficient isolate AH9 was created and tested for its ability to spread on soft agar plates (Fig. 2). This analysis showed that the *sasX* mutant strain covered slightly larger areas, which suggested a trend towards increased spreading motility (Fig. 3). This could be indicative for a spreading-antagonizing role of SasX, similar to that of FnbpA/B and ClfA/B. Nevertheless, despite this apparent phenotype of the *sasX* mutant, the increased spreading was not statistically significant. This result is in accordance with our previous findings, which showed that the absence of only one of the FnbpA, FnbpB, ClfA and ClfB proteins is insufficient for causing a significant increase in spreading motility (19).

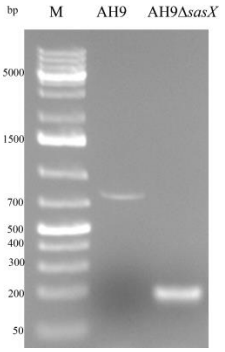

Figure 2. PCR confirmation of the *sasX* deletion from *S. aureus* AH9. The presence of *sasX* in *S. aureus* AH9 and the absence of this gene from the respective *sasX* deletion mutant (AH9 Δ*sasX*) were confirmed by PCR with the *sasX* flanking primers *sasX*-F and *sasX*-R. As expected this resulted in amplified fragments of 790 bp and 175 bp, respectively.

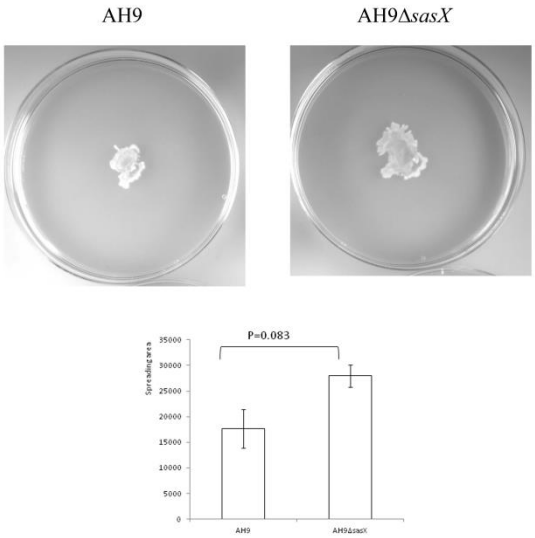

Figure 3. No significant influence of *sasX* deletion on colony spreading by *S. aureus* AH9. The colony spreading analysis, which was repeated three times, included the clinical *S. aureus* isolate AH9 and its *sasX* mutant derivative. The spreading areas of the parental and mutant strains were quantified by image J analysis. Error bars represent the standard deviation (SD). Although there was a trend towards more spreading by the *sasX* mutant, the difference in spreading was not statistically significant (P=0.083).

To further explore a possible role of SasX in colony spreading, we tested whether

overexpression of *sasX* could significantly impact on staphylococcal motility. To this end, *sasX* was ectopically expressed from plasmid sasX-pCN51*cat*. Subsequently, plasmid *sasX*-pCN51*cat* was introduced into strain SH1000Δ, which exhibits a hyper-spreading phenotype due to the absence of FnbpA/B and ClfA/B (19). Notably, expression of *sasX* from the *sasX*-pCN51*cat* plasmid needs to be induced with $CdSO_4$. As shown in Figure 4, addition of this inducer into the soft agar plates resulted in a reduction of colony spreading in all tested strains. Importantly, colony spreading of the SH1000Δ strain expressing *sasX* from the *sasX*-pCN51cat plasmid was slightly decreased compared to the parental strain, but this difference was not statistically significant. We therefore conclude that SasX by itself is not sufficient to antagonize colony spreading as is the case for FnbpA, FnbpB, ClfA and ClfB. Instead, several of these covalently cell wall-attached LPxTG proteins need to work in synergy to limit the colony spreading motility of *S. aureus*.

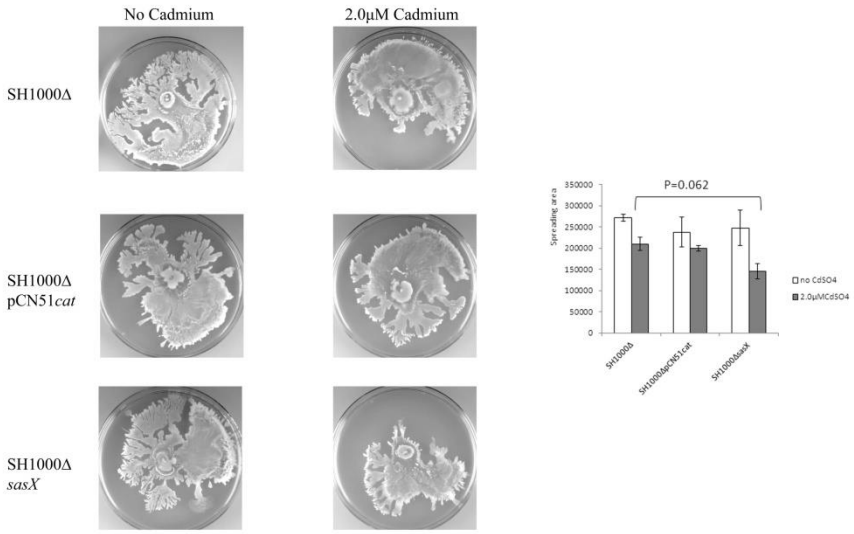

Figure 4. *SasX* expression has no significant influence of on colony spreading. To test the possible influence of *sasX* expression on colony spreading, a plasmid pCN51*cat*-borne copy of *sasX* was expressed in an *S. aureus* mutant lacking the *fnbpA, fnbpB, clfA* and *clfB* genes. (SH1000Δ). The SH1000Δ strain shows a hyper-spreading phenotype due to the absence of FnbpA/B and ClfA/B. Next, the spreading ability of strain SH1000Δ with the *sasX*-pCN51*cat* plasmid, strain SH1000Δ with the empty plasmid pCN51*cat*, and the untransformed SH1000Δ strain was analyzed in the absence of presence of Cadmium. The experiment was repeated three times. The spreading areas were quantified by image J. Error bars represent one standard deviation (SD). There was no statistically significant difference in the spreading abilities of the three investigated strains although the strain expressing *sasX* trended towards a lower spreading ability (P=0.062).

At present, it is not clear how the LPxTG proteins FnbpA, FnbpB, ClfA, ClfB and possibly SasX can antagonize colony spreading. It has been shown that the staphylococcal 'accessory gene regulator' *agr* quorum-sensing system is an important determinant for colony spreading due to its regulatory role on the expression of the phenol soluble modulins (PSMs) (18,20). It is possible that the LPxTG proteins interact with the PSMs thereby blocking or limiting their diffusion on the soft agar plates, which would in turn set a limit to the colony spreading ability of *S. aureus*. Alternatively, the different LPxTG proteins could act synergistically, interacting with each other and/or with other molecules on the surface of neighboring *S. aureus* cells. Such interactions could potentially lead to tighter cell-cell aggregations, which could also limit the ability of the cells to dissociate and move in different directions by making use of the surfactant properties of the PSMs that they secreted. Many LPxTG proteins belong to the so-called MSCRAMMs (microbial surface components recognizing adhesive matrix molecules), which are responsible for bacterial and host interactions (12,15,16). In the absence of these LPxTG proteins, *S. aureus* cannot attach to the host surface, which may enable *S. aureus* cells to colonize different sites within the human host and efficiently transmit among individuals. Interestingly, the processed SasX protein is rather small (~15 kDa), which is likely too small to protrude through the cell wall and expose a domain for interaction (14). This could be another reason why SasX has only a very limited influence on colony spreading, if any. Altogether, the present observations imply that the previously reported influence of SasX on the epidemicity of MRSA in China (11) is probably not exerted via a modulating influence on staphylococcal colony spreading motility.

Reference List

1. Arnaud, M., A. Chastanet, and M. Debarbouille. 2004. New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. Appl.Environ.Microbiol. 70:6887-91.
2. Charpentier, E., A. I. Anton, P. Barry, B. Alfonso, Y. Fang, and R. P. Novick. 2004. Novel cassette-based shuttle vector system for gram-positive bacteria. Appl.Environ.Microbiol. 70:6076-85.
3. Corrigan, R. M., H. Miajlovic, and T. J. Foster. 2009. Surface proteins that promote adherence of *Staphylococcus aureus* to human desquamated nasal epithelial cells. BMC.Microbiol. 9:22.
4. Greene, C., D. McDevitt, P. Francois, P. E. Vaudaux, D. P. Lew, and T. J. Foster. 1995. Adhesion properties of mutants of *Staphylococcus aureus* defective in fibronectin-binding proteins and studies on the expression of *fnb* genes. Mol.Microbiol. 17:1143-52.
5. Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. J.Mol.Biol. 166:557-80.
6. Harshey, R. M. 2003. Bacterial motility on a surface: many ways to a common goal. Annu.Rev.Microbiol. 57:249-73.
7. Inose, Y., S. L. Takeshita, T. Hidaka, M. Higashide, A. Maruyama, H. Hayashi, K. Morikawa, and T. Ohta. 2006. Genetic characterization of the natural SigB variants found in clinical isolates of *Staphylococcus aureus*. J.Gen.Appl.Microbiol. 52:259-71.
8. Kaito, C. and K. Sekimizu. 2007. Colony spreading in *Staphylococcus aureus*. J.Bacteriol. 189:2553-7.
9. Kreiswirth, B. N., S. Lofdahl, M. J. Betley, M. O'Reilly, P. M. Schlievert, M. S. Bergdoll, and R. P. Novick. 1983. The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. Nature 305:709-12.
10. Kuroda, M., R. Ito, Y. Tanaka, M. Yao, K. Matoba, S. Saito, I. Tanaka, and T. Ohta. 2008. *Staphylococcus aureus* surface protein SasG contributes to intercellular autoaggregation of *Staphylococcus aureus*. Biochem.Biophys.Res.Commun. 377:1102-6.
11. Li, M., X. Du, A. E. Villaruz, B. A. Diep, D. Wang, Y. Song, Y. Tian, J. Hu, F. Yu, Y. Lu, and M. Otto. 2012. MRSA epidemic linked to a quickly spreading colonization and virulence determinant. Nat.Med. 18:816-9.
12. Marraffini, L. A., A. C. Dedent, and O. Schneewind. 2006. Sortases and the art of anchoring proteins to the envelopes of gram-positive bacteria. Microbiol.Mol.Biol.Rev. 70:192-221.
13. O'Neill, E., C. Pozzi, P. Houston, H. Humphreys, D. A. Robinson, A. Loughman, T. J. Foster, and J. P. O'Gara. 2008. A novel *Staphylococcus aureus* biofilm phenotype mediated by the fibronectin-binding proteins, FnBPA and FnBPB. J.Bacteriol. 190:3835-50.
14. Otto, M. 2012. Molecular insight into how MRSA is becoming increasingly dangerous. Virulence. 3:521-3.

15. Ponnuraj, K., M. G. Bowden, S. Davis, S. Gurusiddappa, D. Moore, D. Choe, Y. Xu, M. Hook, and S. V. Narayana. 2003. A "dock, lock, and latch" structural model for a staphylococcal adhesin binding to fibrinogen. Cell 115:217-28.

16. Ton-That, H., L. A. Marraffini, and O. Schneewind. 2004. Protein sorting to the cell wall envelope of Gram-positive bacteria. Biochim.Biophys.Acta 1694:269-78.

17. Tsai, M., R. L. Ohniwa, Y. Kato, S. L. Takeshita, T. Ohta, S. Saito, H. Hayashi, and K. Morikawa. 2011. *Staphylococcus aureus* requires cardiolipin for survival under conditions of high salinity. BMC.Microbiol. 11:13.

18. Tsompanidou, E., E. L. Denham, D. Becher, J. A. de, G. Buist, O. M. van, W. L. Manson, J. W. Back, J. M. van Dijl, and A. Dreisbach. 2013. Distinct roles of phenol-soluble modulins in spreading of *Staphylococcus aureus* on wet surfaces. Appl.Environ.Microbiol. 79:886-95.

19. Tsompanidou, E., E. L. Denham, M. J. Sibbald, X. M. Yang, J. Seinen, A. W. Friedrich, G. Buist, and J. M. van Dijl. 2012. The sortase A substrates FnbpA, FnbpB, ClfA and ClfB antagonize colony spreading of *Staphylococcus aureus*. PLoS One 7:e44646.

20. Tsompanidou, E., M. J. Sibbald, M. A. Chlebowicz, A. Dreisbach, J. W. Back, J. M. van Dijl, G. Buist, and E. L. Denham. 2011. Requirement of the *agr* locus for colony spreading of *Staphylococcus aureus*. J.Bacteriol. 193:1267-72.

21. Vergara-Irigaray, M., J. Valle, N. Merino, C. Latasa, B. Garcia, L. M. Ruiz de, I, C. Solano, A. Toledo-Arana, J. R. Penades, and I. Lasa. 2009. Relevant role of fibronectin-binding proteins in *Staphylococcus aureus* biofilm-associated foreign-body infections. Infect.Immun. 77:3978-91.

22. Wertheim, H. F., E. Walsh, R. Choudhurry, D. C. Melles, H. A. Boelens, H. Miajlovic, H. A. Verbrugh, T. Foster, and B. A. van. 2008. Key role for clumping factor B in *Staphylococcus aureus* nasal colonization of humans. PLoS Med. 5:e17.

Chapter 9

Summary and General Discussion

Bacteria are a common cause of infectious diseases and different bacteria occupy different ecological niches, some being obligate pathogens and others more opportunistic, yet others innocuous or even beneficial. Opportunistic bacteria are likely to cause disease in hosts that have underlying diseases, which are causing some type of impairment, either immunologically or anatomically. For humans this means that opportunistic infections are more often seen in hospitals, where patients demand medical attention. Conventional treatment includes antibiotics, and thus human antibiotic exposure is higher in hospitals than in the community, making hospitals the ideal breeding grounds for the selection and emergence of antibiotic resistance. Alternative sources for the emergence of resistant bacteria are farm animals that are produced for human consumption using intensive rearing practices. Since animal densities in these farms are high, infections are frequent and there is an incentive to "protect" farm animals by adding antibiotics to their feed or to use blanket antibiotic coverage for an entire herd if a single individual falls ill, a practice commonly called metaphylaxis. Not surprisingly, there have been various examples that document that antibiotic resistant bacteria or resistance genes, which have emerged in animals, were introduced into humans and eventually also into health care institutions (5,12). *S. aureus* is such a typical example (11). As a facultative (opportunistic) pathogen, *S. aureus* preys on the opportunity to either invade humans causing a broad range of diseases when the primary anatomical barriers are disrupted or to produce toxin-mediated disease.

Typical for *S. aureus* is the rise and fall of certain clonal lineages, which is regularly accompanied by local, regional or global epidemics. These expanding clones often also display multiple antibiotic resistances. As described in **chapter 1** of this thesis, novel lineages of methicillin-resistant *S. aureus* (MRSA) have emerged, leading to a worldwide pandemic of MRSA. There is still speculation that in some instances, the therapeutic pressure exerted by antibiotics may also have affected the disappearance of MRSA thus also terminating epidemic waves (3). From the multiple-resistance healthcare-associated MRSA (HA-MRSA) to high-virulence community-associated MRSA (CA-MRSA) and newly emerging livestock-associated MRSA (LA-MRSA), MRSA clones appear to have acquired phenotypic traits that render them more virulent, resistant, or able to colonize and hence transmit better between different hosts. Knowledge about the nature and the dynamics of MRSA and methicillin-susceptible *S. aureus* (MSSA) clones that are prevalent at different geographic regions is required to implement strategies to control the spread of *S. aureus* in hospitals, the community and animals.

The current thesis describes investigations into the nature and dynamics of *S. aureus* with

emphasis on MRSA in China and draws comparisons with the situation in Europe and beyond. The emergence of MRSA is a worldwide phenomenon, whereby the epidemiological characteristics are changing rapidly. **Chapter 2** describes the occurence of MRSA in a Chinese hospital between 2000 and 2005. Resistance to rifampin increased from 32% to 68%, and the minimal inhibitory concentration that inhibited the growth in 50% of all reported isolates (MIC_{50}) for rifampin increased from 0.008 to 256 mg/ml. Decreasing susceptibility to mupirocin and quinupristin-dalfopristin were also found. Sequence type (ST) ST239-MRSA-III was the predominant clone in 2000 and in 2005, while ST5-MRSA-II was only identified in 2005. The results described in **chapter 2** portray how antimicrobial resistance and clonal composition changes among hospital-associated MRSA isolates over this five years' period. Patients constantly move in and out of hospitals and, as a consequence, some successful community clones could gain a foothold in hospitals while healthcare-associated clones could translocate into communities. In a recent national MRSA survey (13), ST239 was the most prevalent ST, ST5 was the second most common ST nationwide, and importantly, ST59 bearing typical features of CA-MRSA, was identified as an emerging MRSA clone in hospitals in some regions of China. Continuous efforts to understand the changing epidemiology of *S. aureus* infection in humans and animals should therefore be sustained, not only to inform appropriate antimicrobial treatment and effective infection control but also to monitor the evolution and emergence of clones with particular public health importance for early warning and response.

Staphylococcal food-poisoning (SFP) is a frequent cause of food-borne gastroenteritis worldwide. Ninety-four outbreaks of SFP were reported to the National Monitoring Network in 2003-2007, involving 2,223 individuals leading to 1,186 hospitalizations in China. The studies described in **Chapter 3** characterized *S. aureus* strains associated with SFP in Southern China. Two predominant *S. aureus* lineages were identified, corresponding to (i) Pulsed-field gel electrophoresis (PFGE) types A and B with ST6 and (ii) PFGE type C with ST943 which, not unsurprisingly, differ from the *S. aureus* clones typically identified in hospitals, but also from those encountered in animals in China. ST6 strains were recently found in SFP outbreaks in other regions in China (unpublished), which suggested ST6 probably represents a unique ST associated with food products and SFP in China. Further whole-genome sequencing (WGS) will be performed to analyze the origin and phylogeny of SFP-associated ST6 strains. SFP is caused by toxinogenic *S. aureus* strains that express one or more of a family of genes that code for heat-stable enterotoxins. The studies described in **Chapter 3** revealed four staphylococcal enterotoxin (SE) gene profiles, including *sea* (n=45), *sec-seh* (n=3), *seb* (n=2), and *seg-sei* (n=2). Staphylococcal enterotoxin A (SEA) is the most

frequently found toxin among strains causing outbreaks of SFP worldwide. Unlike clones that are found in healthcare-associated infections, which are mainly MRSA, outbreaks of SFP are caused largely by methicillin-susceptible *S. aureus* (MSSA) clones. The majority of the isolates we examined in this study were resistant to only one or two antibiotics. Altogether, the results of the present study will help to improve our understanding of the clonal composition of SFP isolates, which may provide better insights into the epidemiology and outbreaks. Ultimately, this will lead to an improved control of SFP.

In China, pig-associated MRSA isolates have been found to be mainly ST9, whereas ST398 MRSA that is dominant among pigs in Europe (esp. in the Netherlands) (1,11) (4) has not been identified in Chinese slaughter animals. Heilongjiang is an important agricultural province in China. In 2011, 16 million pigs were raised for food production in this province alone. In a study on *S. aureus* carriage among slaughter pigs in this province, it was found that ST398 was the most frequent ST type (99/162, 61.1%) among MSSA, whereas ST398 MRSA was not identified (**chapter 4**). *S. aureus* ST398 is also a frequent cause of community-onset and hospital-acquired infection among humans in China. In Europe, ST398 MRSA is mainly associated with animal origin and is occasionally transmitted to humans via occupational exposure. The real transmission route between humans and animals and the reason why ST398 strains persists as MSSA in China are still unclear and need to be further elucidated. **Chapter 4** also describes the identification of a novel gene, *lsa(E)*, that conveys quinupristin/dalfopristin resistance in *S. aureus* (MRSA as well as MSSA). Isolates harboring this gene were frequently found in pigs and it can be argued that the use of virginiamycin which, in contrast to Europe and the USA, is not banned in China, selects for this trait. The high degree of similarity between the DNA sequence of a novel transposon carrying *lsa(E)* in *S. aureus* and the sequence of plasmid pEF418 of *Enterococcus faecalis* suggests that *S. aureus* acquired this trait by horizontal interspecies gene transfer. All *S. aureus* isolates bearing this novel transposon belonged to ST9, which is indicative of a clonal expansion of this novel resistance trait in pigs. Three ST9 strains (two MRSA and one MSSA) carrying *lsa(E)* were analyzed by WGS (**chapter 7)**. Our phylogenetic analysis based on WGS revealed that the ST9 lineage most probably evolved from a recent common ancestor shared with clonal complex 5. CC5 is one of the most successful healthcare-associated clones, among which vancomycin resistance (VRSA) has repeatedly emerged in the USA. Typically, VRSA is associated with the acquisition of the *vanA* gene located on a Tn1546-containing plasmid also originating from enterococci. On further investigation, we found that all isolates were deficient in the recently described type IV Restriction-Modification (RM) system. As described **in chapter 1,** RM systems are responsible for protecting bacteria from invasion by

foreign DNA. It has been shown that isolates belonging to the CC5 lineage may be deficient in this restriction function, which make them prone to acquire foreign DNA from enterococci. Accordingly, it seems likely that the deficiency of this particular type IV RM system has played a role in the genetic transfer of *lsa(E)* from enterococci to *S. aureus*. Additionally, a type V (5C2)-like SCC*mec* with a class C2 *mec* gene complex and a new allotype *ccrC* gene was identified in two MRSA strains. A 24kb ΨSCC fragment was found both in the MRSA and MSSA isolates sharing 99% nucleotide sequence identity with the ΨSCCJCSC6690 (O-2) element of a ST9 MRSA isolate from Thailand (accession number AB705453). Judged by the outcome of phylogenetic analyses, it is conceivable that the SCC*mec* remnant in the MSSA strain maybe derived from MRSA with loss of the mobile region containing the *mec* and *ccr* gene complexes. Together, these findings indicate that the sequenced quinupristin/dalfopristin resistant ST9 strains represent a reservoir of novel mobile genetic elements associated with new resistance features that may have originated from other species and can now spread into successful human epidemic *S. aureus* lineages.

Additional investigations were aimed at determining the clonal composition of *S. aureus* carried in the Chinese general population (healthy carriage), and risk factors associated with the carriage of particular clones and phenotypes. We therefore investigated 2448 healthy adults (≥18 y) from Beijing (1530) and Harbin (918) (**Chapter 5**) by nasal screening to determine the prevalence and risk factors of *S. aureus* nasal carriage in these two cities from Northern China. We detected the presence of *S. aureus* in 16.5% of our study population, which matches well with the prevalence observed in previous studies in China (2,7). The low prevalence of MRSA (0.36%) and the heterogeneity of *spa* types suggest that there were no singularly expanding MRSA clones among healthy individuals in Northern China. Three factors were independently associated with *S. aureus* nasal carriage, which included city of residence, Harbin (OR=2.0, 95% CI=1.41 to 2.85), age ≤ 24 years (OR=1.77, 95% CI=1.30-2.44) and non-Han ethnicity (OR=1.58, 95% CI=1.05 to 2.38). Based on genetic analysis of the *S. aureus* population using multiple-locus variable number of tandem repeat analysis (MLVA) (see **chapter 6**) and *spa*-typing, the MLVA complex (MC) 398 and MC5a were identified as the most prevalent clonal lineages in this collection. Multivariate models showed that residing in Harbin (OR=1.77, 95% CI=1.07-2.92) and having household members in the healthcare profession (OR=3.69, 95% CI=1.14-11.92) are factors that were independently associated with carriage of the clonal lineage MC398. This result may indicate that healthcare personnel could contribute to the dissemination of MC398 (CC398) strains between hospitals and the community. It would therefore be worthwhile to investigate the transmission dynamics of *S. aureus* CC398 between household members in future studies.

Chapter 6 represents the first preliminary attempt to describe two extant populations of *S. aureus* isolated during dedicated surveys from opposite ends of the same continental shelf – namely China and Europe. In total, 1294 human isolates from China and Europe were characterized by MLVA. Indeed, there were systematic differences in the distribution of major clonal lineages between China and Europe. The five most frequent MCs among the Chinese isolates belonged to MC398, MC5 subclade a, MC8, MC437 and MC7, and these MCs made up 55.0% of the sample. For the European isolates, the five most frequent MCs consisted of MC5 subclade a, MC45, MC8, MC30 and MC22, which accounted for 64.3% of the sample. This result suggests that the Chinese sample consists of a combination of the pandemic lineages CC8/ST239/MC8 and CC5/ST5/MC5, and that there is a considerable expansion of CC398/ST398/MC398. Two main MLVA types (MTs) of the latter lineage were found which were CC398/ST398/MC398/MT565/t571 and MT569/MC398/ST398/CC398/t034. Combined with the results of chapter 4, we suggest that there are two main subclones of MC398 co-circulating in China. Based on purely quantitative inference, i.e. the relative proportions of Chinese vs. European isolates among the founders and their descendants, MC5 subclade b, MC1933, MC398, MC437, MC621, MC1 and MC123 were more likely originating from China whereas MC2, MC22, MC30 and MC45 may have originated in Europe. This study provides a novel hypothesis about the geographical origins of emergence and events leading to the dissemination of clonal lineages of *S. aureus* at continental scales.

The ability of *S. aureus* to move along different natural or artificial surfaces is an important trait in the colonization and invasion of its human host. Additional benefits of this so-called colony spreading motility include increased access to nutrients, avoidance of toxins or the host immune system and, potentially, an increased efficiency of nosocomial transmission from one patient to another. It has been shown that the *agr* quorum-sensing system controls colony spreading by regulating the expression of phenol-soluble modulin (PSM) peptides that promote spreading motility through their strong surfactant properties (8,10). Previous studies have furthermore shown that the spreading motility of *S. aureus* is antagonized by covalently cell wall-bound proteins that mediate cell-cell interactions, like FnbpA, FnbpB, ClfA and ClfB (9). Accordingly, mutant *S. aureus* cells that lack all four of these cell wall proteins show a hyper-spreading phenotype, and the same was observed for mutant cells that lack the transpeptidase sortase A, which is responsible for linking FnbpA, FnbpB, ClfA and ClfB to the cell wall. The φSPβ-like prophage is thought to be an important characteristic of the ST239 'Asian clade' of *S. aureus*, as it carries the *sas*X gene, an important virulence factor that has been implicated as a driving force in the ST239 epidemic (6). Specifically, SasX seems to contribute to virulence and promotes not only the adhesion of *S. aureus* to nasal epithelial

cells, but also intercellular aggregation and biofilm formation. Since SasX is coupled covalently to the cell wall by sortase A, like FnbpA, FnbpB, ClfA and ClfB, the influence of SasX on colony spreading was investigated in the studies described in **chapter 8**. Indeed, an engineered *sasX* mutant strain showed a tendency to cover slightly larger areas than its *sasX*-proficient parental strain. Conversely, a mutant strain lacking *fnbpA*, *fnbpB*, *clfA* and *clfB*, but overexpressing *sasX* trended towards lowered spreading motility. Yet, these observed trends were not statistically significant, which implies that a potential spreading-antagonizing effect of SasX by itself, if any, will be limited. It thus seems that the effects of SasX in the promotion of intercellular aggregation and biofilm formation are not related to a spreading antagonizing activity.

In conclusion, the research described in this thesis has addressed the genetic population structure of *S. aureus* from various origins in China. The collected isolates were compared to representative European strains, and the genetic features of porcine *S. aureus* isolates that carry a novel mobile genetic element conveying antibiotic resistance were analysed in detail. The different studies specifically addressed: (1) the dynamics of HA-MRSA clones in China, (2) community-acquired food-poisoning and health carriage clones, (3) LA-MRSA clones, and (4) genome analysis. One of the main findings was that a single clone, CC398, has spread among livestock, the human population and healthcare facilities in China. Future research should focus on the transmission routes, transmissibility, pathogenicity and evolution of this clone. Another finding described in this thesis is that *S. aureus* ST9 is an important lineage in the exchange of novel MGEs with *Entercoccus* species. Here, the main challenge for future research will be to evaluate the horizontal gene transfer between *S. aureus* and entercocci, or among *S. aureus* lineages. Finally, the present studies raise concern that the use of virginiamycin in livestock-rearing may result in a co-selection for resistance to quinupristin/dalfopristin, a last line antibiotic used for the treatment of infections caused by multiple resistant clones of *S. aureus*. This could further curtail effective antibiotic therapy in humans, and more attention to this potential threat may be warranted by Chinese regulatory bodies.

Reference List

1. de Neeling, A. J., M. J. van den Broek, E. C. Spalburg, M. G. van Santen-Verheuvel, W. D. Dam-Deisz, H. C. Boshuizen, A. W. van de Giessen, D. E. van, and X. W. Huijsdens. 2007. High prevalence of methicillin resistant *Staphylococcus aureus* in pigs. Vet.Microbiol. 122:366-72.
2. Du, J., C. Chen, B. Ding, J. Tu, Z. Qin, C. Parsons, C. Salgado, Q. Cai, Y. Song, Q. Bao, L. Zhang, J. Pan, L. Wang, and F. Yu. 2011. Molecular characterization and antimicrobial susceptibility of nasal *Staphylococcus aureus* isolates from a Chinese medical college campus. PLoS One. 6:e27328.
3. Grundmann, H., M. Aires-de-Sousa, J. Boyce, and E. Tiemersma. 2006. Emergence and resurgence of meticillin-resistant *Staphylococcus aureus* as a public-health threat. Lancet. 368:874-85.
4. Guardabassi, L., M. Stegger, and R. Skov. 2007. Retrospective detection of methicillin resistant and susceptible *Staphylococcus aureus* ST398 in Danish slaughter pigs. Vet.Microbiol. 122:384-6.
5. Klare, I., C. Konstabel, D. Badstubner, G. Werner, and W. Witte. 2003. Occurrence and spread of antibiotic resistances in *Enterococcus faecium*. Int.J.Food Microbiol. 88:269-90.
6. Li, M., X. Du, A. E. Villaruz, B. A. Diep, D. Wang, Y. Song, Y. Tian, J. Hu, F. Yu, Y. Lu, and M. Otto. 2012. MRSA epidemic linked to a quickly spreading colonization and virulence determinant. Nat.Med. 18:816-9.
7. Qu, F., E. Cui, T. Guo, H. Li, S. Chen, L. Liu, W. Han, C. Bao, Y. Mao, and Y. W. Tang. 2010. Nasal colonization of and clonal transmission of methicillin-susceptible *Staphylococcus aureus* among Chinese military volunteers. J.Clin.Microbiol. 48:64-9.
8. Tsompanidou, E., E. L. Denham, D. Becher, J. A. de, G. Buist, O. M. van, W. L. Manson, J. W. Back, J. M. van Dijl, and A. Dreisbach. 2013. Distinct roles of phenol-soluble modulins in spreading of *Staphylococcus aureus* on wet surfaces. Appl.Environ.Microbiol. 79:886-95.
9. Tsompanidou, E., E. L. Denham, M. J. Sibbald, X. M. Yang, J. Seinen, A. W. Friedrich, G. Buist, and J. M. van Dijl. 2012. The sortase A substrates FnbpA, FnbpB, ClfA and ClfB antagonize colony spreading of *Staphylococcus aureus*. PLoS One. 7:e44646.
10. Tsompanidou, E., M. J. Sibbald, M. A. Chlebowicz, A. Dreisbach, J. W. Back, J. M. van Dijl, G. Buist, and E. L. Denham. 2011. Requirement of the *agr* locus for colony spreading of *Staphylococcus aureus*. J.Bacteriol. 193:1267-72.
11. Voss, A., F. Loeffen, J. Bakker, C. Klaassen, and M. Wulf. 2005. Methicillin-resistant *Staphylococcus aureus* in pig farming. Emerg.Infect.Dis. 11:1965-6.
12. Wegener, H. C. 2003. Antibiotics in animal feed and their role in resistance development. Curr.Opin.Microbiol. 6:439-45.
13. Xiao, M., H. Wang, Y. Zhao, L. L. Mao, M. Brown, Y. S. Yu, M. V. O'Sullivan, F. Kong, and Y. C. Xu. 2013. National surveillance of methicillin-resistant *Staphylococcus aureus* in China highlights a still-evolving epidemiology with 15 novel emerging multilocus sequence types. J.Clin.Microbiol. 51:3638-44.

Appendix

Appendix 1: Nederlandse samenvatting

Nederlandse samenvatting

Staphylococcus aureus is een veel voorkomende bacteriële ziekteverwekker bij mens en dier die vaak resistent is tegen antibiotica. De Europese *S. aureus*-populatie is redelijk goed beschreven, maar over de samenstelling van *S. aureus*-populaties in andere delen van de wereld weten we nog betrekkelijk weinig. Het onderzoek beschreven in dit proefschrift geeft eerste inzichten in de populatiestructuur van *S. aureus* in China.

Een eerste onderzoek naar de antibioticumresistentieprofielen van meticilline-resistente *S. aureus* (MRSA), verzameld in een representatief ziekenhuis in Beijing in 2000 en 2005, liet zien dat gedurende deze periode de rifampicine-resistentie van MRSA meer dan verdubbeld was (van 32% naar 68%). Dit onderzoek toonde tevens aan, dat *S. aureus* met het sequentie-type (ST) 239 de dominante kloon was, terwijl het ST5-type pas in 2005 opdook.

Voedselvergiftiging veroorzaakt door *S. aureus* komt vaak voor in China. Daarom werd de epidemiologische context en de genetische verwantschap van *S. aureus*-isolaten uit 11 uitbraken van voedselvergiftiging in Zuid-China in de periode van 2006 tot 2009 onderzocht. Met behulp van pulsed-field gelelectroforese, multilocus sequentie-typering en *spa*-typering werden de twee hoofdverantwoordelijke *S. aureus*-types ST6 en ST943 geïdentificeerd. In alle gevallen ging het om meticilline-gevoelige *S. aureus* (MSSA) isolaten die het hitte-bestendige enterotoxine Sea als belangrijkste virulentiefactor tot expressie brachten.

Om de prevalentie van *S. aureus* in dieren, bestemd voor menselijke consumptie, in China in kaart te brengen werden 590 slachtvarkens onderzocht op nasale kolonisatie in twee slachthuizen in de Noord-Chinese provincie Heilongjiang. Tweehonderd van de onderzochte dieren (33,9%) waren *S. aureus* dragers en 38 (6,4%) droegen MRSA bij zich. Verdere analyses lieten zien, dat de meeste MSSA-isolaten (61,1%) tot het ST398-type behoorden en dat alle MRSA-isolaten tot het ST9-type behoorden. Tevens werd een hoge resistentie tegen quinupristine/dalfopristine waargenomen in 45 *S. aureus*-isolaten, waarbij alle 38 MRSA- en 7 MSSA-isolaten hetzelfde resistentie-fenotype vertoonden. Dit nieuwe resistentie-fenotype was gekoppeld aan de aanwezigheid van het *lsa(E)*-gen in 44 van de onderzochte isolaten die allemaal tot het ST9-type behoorden. Het veelvuldige gebruik van het antibioticum virginiamycine als groeibevorderaar in de Chinese veehouderij en met name de varkenshouderij zou een verklaring kunnen vormen voor de frequente detectie van het *S. aureus* ST9-type met quinupristine/dalfopristine-resistentie in de onderzochte varkens. Van twee MRSA-isolaten en één van de MSSA-isolaten werd het volledige genoom gesequenced. Dit bevestigde de eerdere waarneming, dat het *lsa(E)*-gen gelegen is op een

chromosomaal-geïntegreerd transposon. Tevens werd een type V (5C2)-achtig SCC*mec* element met een klasse C2 *mec*-gencomplex en een nieuw allotype *ccrC*-gen geïdentificeerd in de twee MRSA-isolaten. De genoomanalyse suggereert verder, dat een recent beschreven type IV Restrictie-Modificatie-systeem niet in *S. aureus* ST9 aanwezig is. Samenvattend suggereren de genoomanalyses, dat *S. aureus* ST9 een reservoir van nieuwe mobiele genetische elementen vormt met nieuwe antibioticumresistentiegenen die mogelijk van andere bacteriesoorten afkomstig zijn en die zich wellicht kunnen verspreiden onder *S. aureus*-types die de mens koloniseren.

Verder onderzoek was gericht op de analyse van de *S. aureus*-populatie in gezonde Chinese vrijwilligers en de risicofactoren die geassocieerd zijn met bepaalde klonale lijnen en hun fenotypes. In totaal werden 2448 gezonde volwassenen uit Beijing en Harbin (Heilongjiang provincie) onderzocht op nasale *S. aureus*-kolonisatie. De resultaten lieten zien, dat 16,5% van de onderzochte personen *S. aureus* bij zich droegen, waarbij MRSA met een lage frequentie (0,36%) gedetecteerd werd. Drie risicofactoren voor nasaal *S. aureus*-dragerschap werden geïdentificeerd, te weten woonplaats, leeftijd en een niet-Han etnische afstamming. De CC398- and CC5-types van *S. aureus* werden het meest aangetroffen.

Een vergelijking tussen *S. aureus* stammencollecties uit China en Europa leverde sterke aanwijzingen voor aanzienlijke verschillen in de *S. aureus*-populatiestructuren in beide gebieden. Gebaseerd op de frequentie en verdeling van eerste vertegenwoordigers van bepaalde types en de desbetreffende *S. aureus*-afstammelingen is geprobeerd de geografische oorsprong van verschillende *S. aureus*-types te traceren. De resultaten van deze analyse suggereren, dat sommige types in de oriënt zijn ontstaan, terwijl andere een occidentale oorsprong hebben. Deze bevindingen moeten echter met enige voorzichtigheid geïnterpreteerd worden, omdat verschillen in de manier waarop *S. aureus*-isolaten in China en Europa verzameld werden een vertekend beeld kunnen opleveren, dat niet met behulp van statistische analyses gecorrigeerd kan worden.

Het *S. aureus* ST239-type vormt het meest-voorkomende nosocomiale MRSA-type in China. Recent onderzoek suggereert, dat het succes van dit type gerelateerd is aan de expressie van het covalent celwand-gebonden eiwit SasX, dat zorgt voor de aggregatie van bacteriecellen. Om deze reden werd onderzocht of SasX een mogelijke invloed heeft op de verspreiding van *S. aureus* over natte oppervlakken. Hiertoe werd een *sasX* deletiemutant van *S. aureus* gespot op een zacht agarmedium. De resultaten van deze analyses suggereren, dat SasX geen significante impact heeft op de motiliteit van *S. aureus* ST239 en

dat een andere verklaring gezocht moet worden voor het epidemiologische gedrag van dit *S. aureus*-type.

Samenvattend kan uit de uitgebreide vergelijking van *S. aureus* isolaten uit China en Europa – twee uiteinden van hetzelfde continent – geconcludeerd worden, dat er systematische genetische verschillen zijn tussen de respectievelijke *S. aureus*-populaties. Op grond hiervan konden nieuwe hypotheses opgesteld worden over de geografische oorsprong en de verspreiding van verschillende *S. aureus*-types op continentaal niveau. Deze hypotheses kunnen als leidraad dienen voor toekomstige studies die moeten leiden tot een beter begrip van de evolutie van de bacteriesoort *S. aureus.*

Appendix

Appendix 2: List of publications

List of publications

1. **Xiaomei Yan**, Yanyan Song, Xiaojie Yu, Xiaoxia Tao, Jun Yan, Fengji Luo, Huifang Zhang, Jianfeng Zhang, Qian Li, Lihua He, Shuming Li, Fanliang Meng, Jianzhong Zhang, Hajo Grundmann. Factors associated with *Staphylococcus aureus* nasal carriage among healthy people in Northern China. Clin Microbiol Infect. 2015, 21:157-62.

2. Glasner C, Pluister G, Westh H, Arends JP, Empel J, Giles E, Laurent F, Layer F, Marstein L, Matussek A, Mellmann A, Pérez-Vásquez M, Ungvári E, **Yan X**, Žemličková H, Grundmann H, van Dijl JM. *Staphylococcus aureus spa* type t437: identification of the most dominant community-associated clone from Asia across Europe. Clin Microbiol Infect. 2015, 21:163.e1-8.

3. Xiaomei Yan, Tao Xiaoxia, Yu Xiaojie, Yan Jun, Zhang Jianzhong. Phenotype and genotype of antimicrobial resistance on nasal *Staphylococcus aureus* isolates from healthy people. Chin J Epidemiol, 2015, 36:639-643. [Article in Chinese]

4. **Xiaomei Yan**, Xiaojie Yu, Xiaoxia Tao, Jianfeng Zhang, Binghua Zhang,Rui Dong, Chengyu Xue, Hajo Grundmann, Jianzhong Zhang. *Staphylococcus aureus* ST398 from slaughter pigs in northeast China. Int J Med Microbiol.2014, 304: 379–83.

5. **Xiaomei Yan,** Bing Wang, Xiaoxia Tao, Qinghua Hu, Zhigang Cui, Jianzhong Zhang, Yiman Lin, Yuanhai You, Xiaolu Shi, Hajo Grundmann. Characterization of *Staphylococcus aureus* Strains Associated with Food Poisoning in Shenzhen, China. Appl. Environ. Microbiol. 2012, 78:6637-42.

6. **Xiaomei Yan**, Xiaoxia Tao, Lihua He, Zhigang Cui, Jianzhong Zhang. Increasing resistance in multiresistant methicillin-resistant *Staphylococcus aureus* clones isolated from a Chinese hospital over a five-year period. Journal of Microbial Drug Resistance. 2011, 17:235-9.

7. WANG Yonglu, WANG Duochun, ZHENG Jinxiu, ZHAN Shengwei, TAO Yong, WANG Li, **YAN Xiaomei,** TAO Xiaoxia, ZHANG Jianzhong, KAN Biao. Characteristics of gene typing and drug resistance of *Staphylococcus aureus* in Maanshan, Anhui. China Preventive Medicine. 2011,10:826-9. [Article in Chinese]

8. TAO Xiaoxia, CUI Zhigang, LIU Guodong, **YAN Xiaomei,** ZHANG Jianzhong. Multiple-locus variable-number tandem-repeat analysis of methicillin-resistant *Staphylococcus aureus* isolates from Beijing. Journal of Pathogen Biology. 2010, 5：81-3.

[Article in Chinese]

9. **Xiaomei Yan**, Zongwei Li, Monika A. Chlebowicz, Xiaoxia Tao, Ming Ni, Yuan Hu, Zhen Li, Hajo Grundmann, Xiaochen Bo, Jan Maarten van Dijl, Pengcheng Du, Minli Zhang, Yuanhai You, Fanliang Meng, Shengqi Wang, Jianzhong Zhang. Genetic features of porcine *Staphylococcus aureus* isolates from China that carry the *lsa(E)* gene for quinupristin/dalfopristin resistance.(to be prepared)

10. **Xiaomei Yan,** Zongwei Li, Monika A. Chlebowicz, Xiaoxia Tao, Ming Ni, Yuan Hu, Zhen Li, Hajo Grundmann, Xiaochen Bo, Jan Maarten van Dijl, Pengcheng Du, Minli Zhang, Yuanhai You, Xiaojie Yu, Fanliang Meng, Shengqi Wang, and Jianzhong Zhang. Describing the population structure of *Staphylococcus aureus* in China and Europe by Multiple-Locus Variable Number Tandem Repeat Analysis; clues to geographical origins of emergence and dissemination. (submitted)

11. **Xiaomei Yan,** Eleni Tsompanidou, and Jan Maarten Van Dijl. The influence of SasX on the epidemicity of *Staphylococcus aureus* ST 239 cannot be explained by a role in colony spreading motility. (to be prepared)

Appendix

Appendix 3: Biography

Biography

Education Backgroud

2011-2013 PhD degree, Department of Medical Microbiology, University Medical Center Groningen and University of Groningen, The Netherlands

2002-2005 Master degree, Department of diagnosis for Communicable Disease, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, China.

1994-1999 Bachelor degree, Department of Medical laboratory specialty, Jilin Medical College, China

Working Experience

2005–now Department of diagnosis for Communicable Disease, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, China. In charge of *Staphylococcus aureus* infection disease control and prevention.

1999-2002 Management office, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, China.

Research Interests

Disease control and prevention of infections caused by *S. aureus* and its associated public health affairs like emergency response
Genetic and molecular characterization of *S. aureus*
Mechanisms of antibiotic resistance

Appendix

Appendix 4：Acknowledgements

Acknowledgements

Since I was a young girl, I always dreamed about living and studying in a foreign country. I never thought that it will become true one day. Being a PhD student in Groningen changed my life. I really enjoyed and appreciated it a lot.

First of all, I would like to thank my two promoters and mentors Prof. Zhang Jianzhong and Prof. Hajo Grundmann. I have worked with Prof. Zhang for more than ten years, since I started my Master studies. He is very kind and supported me in any way during these last years. Thank you so much for all your assistance and encouragement. I really appreciated it. My deepest gratitude goes foremost to Prof. Grundmann. Thank you for giving me the opportunity to do my PhD in such a beautiful country. Thank you for your persistent support, encouragement and guidance. I deeply appreciate everything that you have done for me and my family. Thank you so much for taking your time to support my husband as well. Your knowledge about how to publish a scientific paper as a clinical doctor helped him a lot.

I also would like to express my appreciation to my co-promotor, Prof. Jan Maarten van Dijl, who introduced me to the world of basic research. Your knowledge and enthusiasm about science really fascinates me. Thank you for hosting me in your lab, sharing your opinions with me and supporting me to get my own grant in China. I hope that in the future we will get the chance to set up a scientific collaboration. That would be really great.

I would also like to express my gratitude to Prof. Alex Friedrich and Dr. Artur Sabat. The first “Dutch-Chinese Conference on the Epidemiology and Control of Antibiotic Resistance” in China in 2012 was such a success. Thank you so much for your support and for actually making the trip to China. Thanks also to Vika for her work for the conference. I am looking forward to the next time.

Next I would like to thank Leo M. Schouls and Gerlinde N. Pluister from the RIVM for performing the MLVA experiments and analysis for me.

I would like to thank the reading committee, Prof. Robin Köck, Prof. Andreas Voss, and Prof. Ed. Feil for reading and approving this thesis and for their comments.

The biggest thanks I need to give to my three lovely girls, my best friends Dr. Monika A. Chlebowicz, Dr. Eleni Tsompanidou and Dr. Corinna Glasner. Without your intelligent discussions, smart instructions, warmly talking, and carefully checking my thesis, I could not have done without you. I will never forget all of the fun time we had together. Many thanks for being my paranymphs.

A huge thanks to all the members of the MolBac group. Girbe, Jetta, Henrik, Lakshmi, Vahid, Federico, Francisco, Jolanda, Viv, Carmine, Rense, Dennis, Emma, Annette, Ruben and Ewoud for helping me during my stay in your groups. I specially need to mention the two sunshine boys of the lab, Ruben and Ewoud. Ruben, thank you so much for all your smart suggestions. Ewoud, we always had a lot of fun together, which made my PhD life not so boring anymore.

I also like to thank all members of the Medical Microbiology Department who have helped me during these two years. Dr. Tjibbe Donker, thank you for making the nice figure for me. Ank, your help at any time was always more than appreciated.

Of course, I would also like to acknowledge all members of the Department of Diagnosis for Communicable Disease in National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention. Tao Xiaoxia, you are a great assistant and sometimes even my teacher. Without your help, I would not have been able to compile such a huge collection of isolates and to have a total of nine chapters in my thesis. I would also like to thank Zhang Huifang and Meng Fanliang for their help and support in the collection and transportation of my samples. He Lihua, many thanks for planning all the trips and solving any financial issues for me.

I would like to thank all my collaborators. Prof. Yu Xiaojie performed an excellent investigation in Harbin (Heilongjiang Province) and provided all isolates and epidemiology data to me. Prof. Luo Fengji, Dr. Song Yanyan, and Li Qian, thank you for your help in organizing the sample collection at the Chaoyang Center for Disease Control and Prevention in Beijing. The Center for Disease Control and Prevention in Anhui, Shenzhen and Zhejiang and the Beijing Union Medical Hospital also donated *S. aureus* isolates to this thesis. Thank you very much.

My friends in Groningen. Thank you so much for everything. Yuxuan, I am so proud of you. You are such an intelligent and awesome girl. I am so grateful to know you. It was so great that we always encouraged each other, especially during the more depressed times.

I still remember the trip I took to Germany together with Han Jing, Ranran and Ee Soo. That was the most fantastic trip in my life. Four female PhD students spending such a wonderful time together chatting, laughing and playing games while drinking good German beer. It was an awesome night. Thanks girls! Pang Chao, Wu Rui, Wang Rong, and Wei, good luck with your defense.

Most importantly, I would like to thank my dear husband. I am so lucky that I found and married you. You provide me with such a happy, loving and relaxing home. When I want to give up, you always encourage and support me. During the period in the Netherlands you took the initiative to take care of our daughter. Thank you so much for your understanding. My dear daughter Siyu. You are my motivation. Thank you for visiting me in the Netherlands during my PhD. I wish you a wonderful life.

In the end I send my deepest gratitude to my parents and my brother. 谢谢父母赐予我生命，培养我成人。爸爸妈妈，您们辛苦了！正是您们对我毫无保留的支持，才会有我今天可以在自己喜欢的天空自由翱翔。你们的爱是我前行的动力。谢谢您们在我出国初期对我女儿的照顾，我爱你们！哥，谢谢你的鼓励，你是我的榜样，你告诉我有些事情扛一扛就过去了。谢谢嫂子对父母的照顾，得以使我能安心在国外读书！同时也谢谢我们这个大家庭的所有亲朋好友对我的关爱和祝福！

Two years passed by so quickly, but they will be in my heart forever. Thank you all.